

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>C12N 9/40, 15/62, 15/56, A61K 38/47, 48/00, C12N 5/10</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 98/11206</b> <b>(43) International Publication Date:</b> 19 March 1998 (19.03.98)
<b>(21) International Application Number:</b> PCT/US97/16603 <b>(22) International Filing Date:</b> 12 September 1997 (12.09.97) <b>(30) Priority Data:</b> 08/712,614 13 September 1996 (13.09.96) US <b>(71) Applicant (for all designated States except US):</b> TRAN- SKARYOTIC THERAPIES, INC. [US/US]; 195 Albany Street, Cambridge, MA 02139 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SELDEN, Richard, F. [US/US]; 106 Bristol Road, Wellesley, MA 02181 (US). BOROWSKI, Marianne [US/US]; 47 Bartlett Parkway, Winthrop, MA 02152 (US). GILLESPIE, Frances, P. [US/US]; 47 Gleason Road, Lexington, MA 02173 (US). KINOSHITA, Carol, M. [US/US]; 400 Davis Road, Bed- ford, MA 01730 (US). TRECO, Douglas, A. [US/US]; 87 Brantwood Road, Arlington, MA 02174 (US). WILLIAMS, Melanie, D. [US/US]; 7 Malden Street, Natick, MA 01760 (US). <b>(74) Agent:</b> FRASER, Janis, K.; Fish & Richardson PC, 225 Franklin Street, Boston, MA 02110-2804 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> THERAPY FOR $\alpha$ -GALACTOSIDASE A DEFICIENCY  <b>(57) Abstract</b>  A therapeutic method whereby an individual suspected of having an $\alpha$ -galactosidase A deficiency, such as Fabry disease, is treated either with (1) human cells that have been genetically modified to overexpress and secrete human $\alpha$ -gal A, or (2) purified human $\alpha$ -gal A obtained from cultured, genetically modified human cells.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

THERAPY FOR  $\alpha$ -GALACTOSIDASE A DEFICIENCYBackground of the Invention

This invention relates to  $\alpha$ -galactosidase A and  
5 treatment for  $\alpha$ -galactosidase A deficiency.

Fabry disease is an X-linked inherited lysosomal storage disease characterized by symptoms such as severe renal impairment, angiokeratomas, and cardiovascular abnormalities, including ventricular enlargement and  
10 mitral valve insufficiency. The disease also affects the peripheral nervous system, causing episodes of agonizing, burning pain in the extremities. Fabry disease is caused by a deficiency in the enzyme  $\alpha$ -galactosidase A ( $\alpha$ -gal A), which results in a blockage of the catabolism of  
15 neutral glycosphingolipids, and accumulation of the enzyme's substrate, ceramide trihexoside, within cells and in the bloodstream.

Due to the X-linked inheritance pattern of the disease, essentially all Fabry disease patients are male.  
20 Although a few severely affected female heterozygotes have been observed, female heterozygotes are generally either asymptomatic or have relatively mild symptoms largely limited to a characteristic opacity of the cornea. An atypical variant of Fabry disease, exhibiting  
25 low residual  $\alpha$ -gal A activity and either very mild symptoms or apparently no other symptoms characteristic of Fabry disease, correlates with left ventricular hypertrophy and cardiac disease (Nakano et al., New Engl. J. Med. 333:288-293, 1995). It has been speculated that  
30 reduction in  $\alpha$ -gal A may be the cause of such cardiac abnormalities.

The cDNA and gene encoding human  $\alpha$ -gal A have been isolated and sequenced (Bishop et al., Proc. Natl. Acad. Sci. USA 83:4859, 1986; Kornreich et al., Nuc. Acids Res.  
35 17:3301, 1988; Oeltjen et al., Mammalian Genome 6:335-

- 2 -

338, 1995). Human  $\alpha$ -gal A is expressed as a 429-amino acid polypeptide, of which the N-terminal 31 amino acids constitute a signal peptide. The human enzyme has been expressed in Chinese Hamster Ovary (CHO) cells (Desnick, 5 U.S. Patent No. 5,356,804; Ioannou et al., J. Cell Biol. 119:1137, 1992); insect cells (Calhoun et al., U.S. Patent No. 5,179,023); and COS cells (Tsuji et al., Eur. J. Biochem. 165:275, 1987). Pilot trials of  $\alpha$ -gal A replacement therapies have been reported, using protein 10 derived from human tissues (Mapes et al., Science 169:987 1970; Brady et al., N. Engl. J. Med. 289:9, 1973; Desnick et al., Proc. Natl. Acad. Sci. USA 76:5326, 1979), but there is currently no effective treatment for Fabry disease.

15                   Summary of the Invention

It has been found that expressing a DNA encoding human  $\alpha$ -gal A in cultured human cells produces a polypeptide that is glycosylated appropriately, so that it is not only enzymatically active and capable of acting 20 on the glycosphingolipid substrate which accumulates in Fabry disease, but is also efficiently internalized by cells via cell surface receptors which target it exactly to where it is needed in this disease: the lysosomal compartment of affected cells, particularly the 25 endothelial cells lining the patient's blood vessels. This discovery, which is discussed in more detail below, means that an individual suspected of having an  $\alpha$ -gal A deficiency such as Fabry disease can be treated either with (1) human cells that have been genetically modified 30 to overexpress and secrete human  $\alpha$ -gal A, or (2) purified human  $\alpha$ -gal A obtained from cultured, genetically modified human cells.

Therapy via the first route, i.e., with the modified cells themselves, involves genetic manipulation



- 3 -

of human cells (e.g., primary cells, secondary cells, or immortalized cells) *in vitro* or *ex vivo* to induce them to express and secrete high levels of human  $\alpha$ -gal A, followed by implantation of the cells into the patient, as generally described in Selden et al., WO 93/09222 (herein incorporated by reference).

When cells are to be genetically modified for the purposes of treatment of Fabry disease by either gene therapy or enzyme replacement therapy, a DNA molecule that contains an  $\alpha$ -gal A cDNA or genomic DNA sequence may be contained within an expression construct and introduced into primary or secondary human cells (e.g., fibroblasts, epithelial cells including mammary and intestinal epithelial cells, endothelial cells, formed elements of the blood including lymphocytes and bone marrow cells, glial cells, hepatocytes, keratinocytes, muscle cells, neural cells, or the precursors of these cell types) by standard methods of transfection including, but not limited to, liposome-, polybrene-, or DEAE dextran-mediated transfection, electroporation, calcium phosphate precipitation, microinjection, or velocity driven microprojectiles ("biolistics"). Alternatively, one could use a system that delivers DNA by viral vector. Viruses known to be useful for gene transfer include adenoviruses, adeno associated virus, herpes virus, mumps virus, poliovirus, retroviruses, Sindbis virus, and vaccinia virus such as canary pox virus. Although primary or secondary cell cultures are preferred for the therapy methods of the invention, one can also use immortalized human cells. Examples of immortalized human cell lines useful in the present methods include, but are not limited to, Bowes Melanoma cells (ATCC Accession No. CRL 9607), Daudi cells (ATCC Accession No. CCL 213), HeLa cells and derivatives of HeLa cells (ATCC Accession Nos. CCL 2, CCL 2.1, and

- 4 -

CCL 2.2), HL-60 cells (ATCC Accession No. CCL 240), HT1080 cells (ATCC Accession No. CCL 121), Jurkat cells (ATCC Accession No. TIB 152), KB carcinoma cells (ATCC Accession No. CCL 17), K-562 leukemia cells (ATCC Accession No. CCL 243), MCF-7 breast cancer cells (ATCC Accession No. BTH 22), MOLT-4 cells (ATCC Accession No. 1582), Namalwa cells (ATCC Accession No. CRL 1432), Raji cells (ATCC Accession No. CCL 86), RPMI 8226 cells (ATCC Accession No. CCL 155), U-937 cells (ATCC Accession No. CRL 1593), WI-38VA13 subline 2R4 cells (ATCC Accession No. CLL 75.1), and 2780AD ovarian carcinoma cells (Van der Blick et al., Cancer Res. 48:5927-5932, 1988) as well as heterohybridoma cells produced by fusion of human cells and cells of another species. Secondary human fibroblast strains, such as WI-38 (ATCC Accession No. CCL 75) and MRC-5 (ATCC Accession No. CCL 171), may also be used.

Following the genetic engineering of human cells with a DNA molecule encoding  $\alpha$ -gal A (or following another appropriate genetic modification, as described below) to produce a cell which overexpresses and secretes  $\alpha$ -gal A, a clonal cell strain consisting essentially of a plurality of genetically identical cultured primary human cells, or, where the cells are immortalized, a clonal cell line consisting essentially of a plurality of genetically identical immortalized human cells, may be generated. Preferably, the cells of the clonal cell strain or clonal cell line are fibroblasts.

The genetically modified cells can then be prepared and introduced into the patient by appropriate methods, e.g. as described in Selden et al., WO 93/09222.

Gene therapy in accordance with the invention possesses a number of advantages over enzyme replacement therapy with enzyme derived from human or animal tissues. For example, the method of the invention does not depend

- 5 -

upon the possibly inconsistent availability of sources of appropriate tissues, and so is a commercially viable means of treating  $\alpha$ -gal A deficiency. It is relatively risk-free compared to enzyme-replacement therapy with enzyme derived from human tissues, which may be infected with known or unknown viruses and other infective agents. Furthermore, gene therapy in accordance with the invention possesses a number of advantages over enzyme replacement therapy in general. For example, the method of the invention (1) provides the benefits of a long-term treatment strategy that eliminates the need for daily injections; (2) eliminates the extreme fluctuations in serum and tissue concentrations of the therapeutic protein, which typically accompany conventional pharmacologic delivery; and (3) is likely to be less expensive than enzyme replacement therapy because production and purification of the protein for frequent administration are unnecessary.

As described above, individuals with  $\alpha$ -gal A deficiencies may also be treated with purified  $\alpha$ -gal A (i.e. enzyme replacement therapy). Primary, secondary, or immortalized human cells genetically modified to overexpress human  $\alpha$ -gal A will also be useful for *in vitro* protein production, to produce protein which may be purified for enzyme replacement therapy. Secondary or immortalized human cells may be chosen from among those described above and may be genetically modified by the transfection or transduction methods also described above. After genetic modification, the cells are cultured under conditions permitting overexpression and secretion of  $\alpha$ -gal A. The protein is isolated from the cultured cells by collecting the medium in which the cells are grown, and/or lysing the cells to release their contents, and then applying standard protein purification techniques. One such technique involves passing the

- 6 -

culture medium, or any sample containing human  $\alpha$ -gal A, over a hydrophobic interaction resin such as Butyl Sepharose® or another resin having a functional moiety that includes a butyl group. Passing the sample over  
5 such a resin may constitute the first chromatography step. If further purification is required, the  $\alpha$ -gal A-containing material eluted from the hydrophobic interaction resin may be passed over a column containing a second resin, such as an immobilized heparin resin such  
10 as Heparin Sepharose®, hydroxyapatite, an anion exchange resin such as Q Sepharose®, or a size exclusion resin such as Superdex® 200. Preferably, the purification protocol would include use of each of the above types of resins. Alternatively, one could use one or more of the  
15 latter resins before or instead of the hydrophobic interaction resin.

Previous methods for the preparation of  $\alpha$ -gal A with relatively high purity were dependent on the use of affinity chromatography, using a combination of lectin  
20 affinity chromatography (concanavalin A (Con A) Sepharose) and affinity chromatography based on binding of  $\alpha$ -gal A to the substrate analog N-6-aminohexanoyl- $\alpha$ -D-galactosylamine coupled to a Sepharose matrix (Bishop et al., J. Biol. Chem. 256:1307-1316, 1981). The use of  
25 proteinaceous lectin affinity resins and substrate analog resins is typically associated with the continuous leaching of the affinity agent from the solid support (cf. Marikar et al., Anal. Biochem. 201:306-310, 1992), resulting in contamination of the purified product with  
30 the affinity agent either free in solution or bound to eluted protein. Such contaminants make the product unsuitable for use in pharmaceutical preparations. Bound substrate analogs and lectins can also have substantial negative effects on the enzymatic, functional, and  
35 structural properties of proteins. Furthermore, such

- 7 -

affinity resins are typically expensive to prepare, making the use of such resins less suitable for production on a commercial scale than more conventional chromatography resins. Thus, the development of a  
5 purification protocol using conventional chromatography resins, which are readily available in supplies and quality suitable for large-scale commercial use, is a significant advantage of the present invention.

An individual who is suspected of having an  $\alpha$ -gal A deficiency may be treated by administration of  
10 pharmaceutically acceptable, purified human  $\alpha$ -gal A by any standard method, including but not limited to intravenous, subcutaneous, or intramuscular injection, or as a solid implant. The purified protein may be  
15 formulated in a therapeutic composition consisting of an aqueous solution containing a physiologically acceptable excipient, e.g. a carrier such as human serum albumin, at pH 6.5 or below.

The present invention thus provides a means for  
20 obtaining large quantities of appropriately glycosylated and therefore therapeutically useful human  $\alpha$ -gal A. This makes enzyme replacement therapy for  $\alpha$ -gal deficiency commercially viable, as well as relatively risk-free compared to therapy with enzyme derived from human or  
25 animal tissues.

Skilled artisans will recognize that the human  $\alpha$ -gal A DNA sequence (either cDNA or genomic DNA), or sequences that differ from it due to either silent codon changes or to codon changes that produce conservative  
30 amino acid substitutions, can be used to genetically modify cultured human cells so that they will overexpress and secrete the enzyme. It is also possible that certain mutations in the  $\alpha$ -gal A DNA sequence will encode polypeptides that retain or exhibit improved  $\alpha$ -gal A  
35 enzymatic activity (as would be apparent by expressing

- 8 -

the mutant DNA molecule in cultured cells, purifying the encoded polypeptide, and measuring the catalytic activity, as described herein). For example, one would expect conservative amino acid substitutions to have  
5 little or no effect on the biological activity, particularly if they represent less than 10% of the total number of residues in the protein. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine,  
10 leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

Production of  $\alpha$ -gal A by the cells can be maximized by certain genetic manipulations. For example,  
15 the DNA molecule that encodes  $\alpha$ -gal A may also encode an heterologous signal peptide, such as the signal peptide of human growth hormone (hGH), erythropoietin, Factor VIII, Factor IX, glucagon, the low density lipoprotein (LDL) receptor, or a lysosomal enzyme other  
20 than  $\alpha$ -gal A. Preferably, the signal peptide is the hGH signal peptide (SEQ ID NO:21), and is at the N-terminus of the encoded protein. The DNA sequence encoding the signal peptide may contain an intron such as the first intron of the hGH gene, resulting in a DNA sequence  
25 such as SEQ ID NO:27 (see also Fig. 10). Furthermore, the DNA molecule may also contain a 3' untranslated sequence (UTS) that is at least 6 nucleotides in length (in contrast to the  $\alpha$ -gal A mRNA found in humans, which has no 3' UTS, the requisite polyadenylation site being  
30 within the coding sequence). The UTS is positioned immediately 3' to the termination codon of the coding sequence, and includes a polyadenylation site. It is preferably at least 6 nucleotides in length, more preferably at least 12, and most preferably at least 30,  
35 and in all cases it contains the sequence AATAAA or a

- 9 -

related sequence which serves to promote polyadenylation. A DNA molecule as described, i.e., encoding an hGH signal peptide linked to  $\alpha$ -gal A and containing a 3' UTS that includes a polyadenylation site, and preferably including  
5 expression control sequences, is also within the invention. Also within the scope of the invention is a DNA molecule encoding a protein that includes the signal peptide of hGH linked to  $\alpha$ -gal A or any other heterologous polypeptide (i.e., any polypeptide other  
10 than hGH or an analog of hGH). The heterologous polypeptide is typically a mammalian protein, e.g. any medically desirable human polypeptide.

Other features and advantages of the invention will be apparent from the detailed description that  
15 follows, and from the claims.

The term "genetically modified," as used herein in reference to cells, is meant to encompass cells that express a particular gene product following introduction of a DNA molecule encoding the gene product and/or  
20 regulatory elements that control expression of a coding sequence. The introduction of the DNA molecule may be accomplished by gene targeting (i.e., introduction of a DNA molecule to a particular genomic site); furthermore homologous recombination allows replacement of the  
25 defective gene itself (the defective  $\alpha$ -gal A gene or a portion of it could be replaced in a Fabry disease patient's own cells with the whole gene or a portion thereof)).

The term " $\alpha$ -gal A," as used herein, means  $\alpha$ -gal A  
30 without a signal peptide, i.e., SEQ ID NO:26 (Fig. 9). There is some indication that residues 371 to 398 or 373 to 398 of SEQ ID NO:26 (Fig. 9) may be removed in the lysosome; however, removal of this putative propeptide is not believed to affect activity of the enzyme. This  
35 suggests that any portion of the putative propeptide

- 10 -

could be deleted without affecting activity. Thus, the term " $\alpha$ -gal A" as used herein also covers a protein having a sequence corresponding to SEQ ID NO:26 except lacking up to 28 residues at the C-terminus of that  
5 sequence.

By " $\alpha$ -gal A deficiency" is meant any deficiency in the amount or activity of this enzyme in a patient. The deficiency may induce severe symptoms as typically observed in males who are suffering from Fabry disease,  
10 or may be only partial and induce relatively mild symptoms as can be seen in heterozygous female carriers of the defective gene.

As used herein, the term "primary cell" includes cells present in a suspension of cells isolated from a  
15 vertebrate tissue source (prior to their being plated, i.e., attached to a tissue culture substrate such as a dish or flask), cells present in an explant derived from tissue, both of the previous types of cells plated for the first time, and cell suspensions derived from these  
20 plated cells.

"Secondary cells" refers to cells at all subsequent steps in culturing. That is, the first time a plated primary cell is removed from the culture substrate and replated (passaged), it is referred to as a secondary  
25 cell, as are all cells in subsequent passages.

A "cell strain" consists of secondary cells which have been passaged one or more times; exhibit a finite number of mean population doublings in culture; exhibit the properties of contact-inhibited, anchorage dependent  
30 growth (except for cells propagated in suspension culture); and are not immortalized.

By "immortalized cell" is meant a cell from an established cell line that exhibits an apparently unlimited lifespan in culture.



- 11 -

By "signal peptide" is meant a peptide sequence that directs a newly synthesized polypeptide to which it is attached to the endoplasmic reticulum for further post-translational processing and/or distribution.

5       The term "heterologous signal peptide," as used herein in the context of  $\alpha$ -gal A, means a signal peptide that is not the human  $\alpha$ -gal A signal peptide (i.e., that encoded by nucleotides 36-128 of SEQ ID NO:18). It typically is the signal peptide of some mammalian protein  
10 other than  $\alpha$ -gal A.

The term "first chromatography step" refers to the first application of a sample to a chromatography column (all steps associated with the preparation of the sample are excluded).

15       Brief Description of the Drawings

Fig. 1 is a representation of the 210 bp probe that was used to isolate  $\alpha$ -gal A from a human fibroblast cDNA library (SEQ ID NO:19). The sequence is from exon 7 of the  $\alpha$ -gal A gene. The probe was isolated from human  
20 genomic DNA by the polymerase chain reaction (PCR). The regions underlined in the figure correspond to the sequences of the amplification primers.

Fig. 2 is a representation of the sequence of the DNA fragment that completes the 5' end of the  $\alpha$ -gal A  
25 cDNA clone (SEQ ID NO:20). This fragment was amplified from human genomic DNA by PCR. The regions underlined correspond to the sequences of the amplification primers. The positions of the NcoI and SacII restriction endonuclease sites, which were used for subcloning as  
30 described in Example IA, are also shown.

Fig. 3 is a representation of the sequence of  $\alpha$ -gal A cDNA, including the sequence that encodes the signal peptide (SEQ ID NO:18).

Fig. 4 is a schematic map of pXAG-16, an  $\alpha$ -gal A  
35 expression construct that includes the

- 12 -

CMV (cytomegalovirus) promoter and intron, the hGH signal peptide coding sequence and first intron, the cDNA for  $\alpha$ -gal A (i.e., lacking the  $\alpha$ -gal A signal peptide sequence) and the hGH 3' UTS.

5            Fig. 5 is a schematic map of pXAG-28, an  $\alpha$ -gal A expression construct that includes the collagen Ia2 promoter, a  $\beta$ -actin intron, the hGH signal peptide coding sequence and first intron, the cDNA for  $\alpha$ -gal A (i.e., lacking the  $\alpha$ -gal A signal peptide sequence) and the hGH  
10 3' UTS.

Fig. 6 is a chromatogram of the  $\alpha$ -gal A purification step using Butyl Sepharose® resin. The absorbance at 280 nm (plain line) and  $\alpha$ -gal A activity (dotted line) of selected fractions is shown.

15            Fig. 7 is a line graph depicting internalization by Fabry fibroblasts of human  $\alpha$ -gal A prepared in accordance with the invention. The intracellular  $\alpha$ -gal A activity and total protein concentration were measured after incubation of the cells with increasing  
20 concentrations of human  $\alpha$ -gal A prepared in accordance with the invention. The effects of the potential internalization-inhibitors mannose-6 phosphate (M6P; open diamonds) and mannan (open circles) are shown.

Fig. 8 is a schematic diagram of the experimental  
25 paradigm designed to examine Fabry fibroblasts following internalization of  $\alpha$ -gal A. The  $\alpha$ -gal A activity of the Fabry cells is measured after exposure to either normal or  $\alpha$ -gal A-overexpressing human fibroblasts cultured in Transwell® inserts. "M6P" = mannose-6 phosphate;  
30 "Untrf HF" = untransfected human fibroblasts; "BRS11" = a transfected,  $\alpha$ -gal A-overexpressing fibroblast strain.

Fig. 9 is a representation of the human  $\alpha$ -gal A amino acid sequence (SEQ ID NO:26).

- 13 -

Fig. 10 is a representation of the DNA sequence encoding the hGH signal peptide and containing the first hGH intron (underlined) (SEQ ID NO:27).

Fig. 11 is a representation of the DNA sequence  
5 encoding the hGH signal peptide without the intron (SEQ ID NO:22).

Fig. 12 is a representation of the amino acid sequence of the hGH signal peptide (SEQ ID NO:21).

Fig. 13 is a representation of the cDNA sequence  
10 encoding human  $\alpha$ -gal A (without signal peptide) (SEQ ID NO:25).

#### Detailed Description

Lysosomal enzymes such as  $\alpha$ -gal A are targeted to the lysosomal compartment of a cell through interaction  
15 with the mannose-6-phosphate (M6P) receptor, which binds to M6P residues present in the oligosaccharide moieties of enzymes destined for the lysosomal compartment (Kornfeld, S. and Mellman, I, Ann. Rev. Cell Biol. 5:483-525, 1989). The primary interaction occurs in the Golgi,  
20 where enzymes bound to Golgi M6P receptors are segregated for transport to the lysosomes. A secondary type of interaction is believed to take place between extracellular  $\alpha$ -gal A and M6P receptors at the cell surface. Extracellular substances internalized by cells  
25 are transported through the cytoplasm in endocytic vesicles, which fuse with primary lysosomes and empty their contents into the lysosomes. In this process, cell surface M6P receptors are also incorporated into endocytic vesicles and transported to lysosomes.

30 Any  $\alpha$ -gal A present in the extracellular milieu can, if it bears M6P residues, bind to the cell surface M6P receptors and thereby be transported into the lysosomal compartment along with the receptors. Once in the lysosomal compartment as a result of this scavenger

- 14 -

pathway, the enzyme can carry out its appropriate function. Thus, even if a cell is genetically deficient in producing its own  $\alpha$ -gal A, there exists a mechanism for it to take up exogenously produced enzyme, provided  
5 that (a) the enzyme is suitably glycosylated and (b) the deficient cell bears M6P receptors.

In Fabry disease, vascular endothelial cells of the kidney and heart have been shown to display severe histopathologic abnormalities and contribute to the  
10 clinical pathology of the disease; these cells, which do carry M6P receptors, are a particular target of the presently claimed invention. The  $\alpha$ -gal A produced in accordance with the invention may be delivered either locally or systemically to the affected cells, by gene  
15 therapy (i.e., by genetically modified cells which express and secrete the glycosylated enzyme within the patient), or by conventional pharmacologic routes of administration. An  $\alpha$ -gal A in which M6P is present in the N-linked oligosaccharides is therefore of great  
20 importance for therapy in accordance with the invention.

Furthermore, the degree to which the N-linked oligosaccharides of  $\alpha$ -gal A are modified by sialylation is also of great importance. In the absence of appropriate sialylation,  $\alpha$ -gal A will be rapidly cleared  
25 from the circulation due to binding by hepatic asialoglycoprotein receptors, followed by internalization and degradation by hepatocytes (Ashwell and Harford, Ann. Rev. Biochem. 51:531-554, 1982). This decreases the amount of  $\alpha$ -gal A available in the circulation for  
30 binding to M6P receptors on cells which contribute to the clinical pathology of Fabry disease, such as the vascular endothelial cells of the kidney and heart. Surprisingly, the Applicants have found that  $\alpha$ -gal A secreted by stably transfected human cells has glycosylation properties  
35 which are suitable for the treatment of Fabry disease by

- 15 -

either gene therapy or by conventional pharmaceutical administration of the purified secreted protein. This is in contrast to the situation with the best-studied lysosomal enzyme, glucocerebrosidase, in which delivery of the enzyme purified from human placenta or secreted from transfected CHO cells to the clinically-relevant cells in the body requires complex enzymatic modification of the enzyme following purification (cf. Beutler, New Engl. J. Med. 325:1354-1360, 1991).

10       The therapy of the invention can be carried out in either of two general ways: by introducing into the patient a therapeutically effective amount of purified human  $\alpha$ -gal A obtained from cultured human cells genetically modified to overexpress and secrete the enzyme, or by introducing the overexpressing cell itself into the patient. Techniques to accomplish the necessary genetic modifications are discussed below, as are the methods of purification, formulation, and treatment.

20       Example I. Preparation and Use of Constructs Designed to Deliver and Express  $\alpha$ -Gal A

Two expression plasmids, pXAG-16 and pXAG-28, were constructed. These plasmids contain human  $\alpha$ -gal A cDNA encoding the 398 amino acids of the  $\alpha$ -gal A enzyme (without the  $\alpha$ -gal A signal peptide); the human growth hormone (hGH) signal peptide genomic DNA sequence, which is interrupted by the first intron of the hGH gene; and the 3' untranslated sequence (UTS) of the hGH gene, which contains a signal for polyadenylation. Plasmid pXAG-16 has the human cytomegalovirus immediate-early (CMV IE) promoter and first intron (flanked by non-coding exon sequences), while pXAG-28 is driven by the collagen I $\alpha$ 2 promoter and also contains the  $\beta$ -actin gene's 5' UTS, which contains the first intron of the  $\beta$ -actin gene.

- 16 -

A. Cloning of the Complete  $\alpha$ -Gal A  
cdNA, and Construction of the  $\alpha$ -Gal A  
Expression Plasmid pXAG-16

The human  $\alpha$ -gal cdNA was cloned from a human  
5 fibroblast cdNA library that was constructed as follows.  
Poly-A<sup>+</sup> mRNA was isolated from total RNA, and cdNA  
synthesis was performed using reagents for the  
lambda ZapII® system according to the manufacturer's  
instructions (Stratagene Inc., LaJolla, CA). Briefly,  
10 "first strand" cdNA was generated by reverse  
transcription in the presence of an oligo-dT primer  
containing an internal XhoI restriction endonuclease  
site. Following treatment with RNase H, the cdNA was  
nick-translated with DNA polymerase I to generate double  
15 stranded cdNA. This cdNA was made blunt-ended with  
T4 DNA polymerase, and ligated to EcoRI adaptors. The  
products of this ligation were treated with T4 DNA kinase  
and digested with XhoI. The cdNA was fractionated by  
Sephacryl-400® chromatography. Large and medium size  
20 fractions were pooled and the cDNAs ligated to EcoRI and  
XhoI-digested Lambda ZapII arms. The products of this  
ligation were then packaged and titered. The primary  
library had a titer of  $1.2 \times 10^7$  pfu/ml and an average  
insert size of 925 bp.

25 A 210 bp probe from exon 7 of the human  $\alpha$ -gal A  
gene (Fig. 1, SEQ ID NO:19) was used to isolate the cdNA.  
The probe itself was isolated from genomic DNA by the  
polymerase chain reaction (PCR) using the following  
oligonucleotides: 5'-CTGGGCTGTAGCTATGATAAAC-3' (Oligo 1;  
30 SEQ ID NO:1) and 5'-TCTAGCTGAAGCAAAACAGTG-3' (Oligo 2;  
SEQ ID NO:2). The PCR product was then used to screen  
the fibroblast cdNA library, and positive clones were  
isolated and further characterized. One positive clone,  
phage 3A, was subjected to the lambda ZapII® system  
35 excision protocol (Stratagene, Inc., La Jolla, CA),

- 17 -

according to the manufacturer's instructions. This procedure yielded plasmid pBSAG3A, which contains the  $\alpha$ -gal A cDNA sequence in the pBluescriptSK-<sup>™</sup> plasmid backbone. DNA sequencing revealed that this plasmid did not contain the complete 5' end of the cDNA sequence. Therefore, the 5' end was reconstructed using a PCR fragment amplified from human genomic DNA. To accomplish this, a 268 bp genomic DNA fragment (Fig. 2, SEQ ID NO:20) was amplified using the following

10 oligonucleotides: 5'-ATTGGTCCGCCCCTGAGGT-3' (Oligo 3; SEQ ID NO:3) and 5'-TGATGCAGGAATCTGGCTCT-3' (Oligo 4; SEQ ID NO:4). This fragment was subcloned into a "TA" cloning plasmid (Invitrogen Corp., San Diego, CA) to generate plasmid pTAAGEI. Plasmid pBSAG3A, which

15 contains the majority of the  $\alpha$ -gal A cDNA sequence, and pTAAGEI, which contains the 5' end of the  $\alpha$ -gal A cDNA, were each digested with SacII and NcoI. The positions of the relevant SacII and NcoI sites within the amplified DNA fragment are shown in Fig. 2. The 0.2 kb SacII-NcoI

20 fragment from pTAAGEI was isolated and ligated to equivalently digested pBSAG3A. This plasmid, pAGAL, contains the complete  $\alpha$ -gal A cDNA sequence, including the sequence encoding the  $\alpha$ -gal A signal peptide. The cDNA was completely sequenced (shown in Fig. 3 including

25 the  $\alpha$ -gal A signal peptide; SEQ ID NO: 18) and found to be identical to the published sequence for the human  $\alpha$ -gal A cDNA (Genbank sequence HUMGALA).

The plasmid pXAG-16 was constructed via several intermediates, as follows. First, pAGAL was digested

30 with SacII and XhoI and blunt-ended. Second, the ends of the complete  $\alpha$ -gal A cDNA were ligated to XbaI linkers and subcloned into XbaI digested pEF-BOS (Mizushima et al., Nucl. Acids Res. 18:5322, 1990), creating pXAG-1. This construct contains the human granulocyte-colony

35 stimulating factor (G-CSF) 3' UTS and the human

- 18 -

elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) promoter flanking the cDNA encoding  $\alpha$ -gal A plus the  $\alpha$ -gal A signal peptide, such that the 5' end of the  $\alpha$ -gal A cDNA is fused to the EF-1 $\alpha$  promoter. To create a construct with the CMV IE promoter and first intron, the  $\alpha$ -gal A cDNA and G-CSF 3' UTS were removed from pXAG-1 as a two kb XbaI-BamHI fragment. The fragment was blunt-ended, ligated to BamHI linkers, and inserted into BamHI digested pCMVflpNeo (which was constructed as described below). The orientation was such that the 5' end of the  $\alpha$ -gal A cDNA was fused to the CMV IE promoter region.

pCMVflpNeo was created as follows. A CMV IE gene promoter fragment was amplified by PCR using CMV genomic DNA as a template and the oligonucleotides:

5'-TTTGGATCCCTCGAGGACATTGATTATTGACTAG-3' (SEQ ID NO:23)  
and 5'-TTTGGATCCCGTGTCAAGGACGGTGAC-3' (SEQ ID NO:24).

The resulting product (a 1.6 kb fragment) was digested with BamHI, yielding a CMV promoter-containing fragment with cohesive BamHI-digested ends. The neo expression unit was isolated from plasmid pMC1neoPA (Stratagene Inc., La Jolla, CA) as a 1.1 kb XhoI-BamHI fragment. The CMV promoter-containing and neo fragments were inserted into a BamHI-, XhoI-digested plasmid (pUC12). Notably, pCMVflpNeo contains the CMV IE promoter region, beginning at nucleotide 546 and ending at nucleotide 2105 (of Genbank sequence HS5MIEP), and the neomycin resistance gene driven by the Herpes Simplex Virus (HSV) thymidine kinase promoter (the TKneo gene) immediately 5' to the CMV IE promoter fragment. The direction of transcription of the neo gene is the same as that of the CMV promoter fragment. This intermediate construct was called pXAG-4.

To add the hGH 3' UTS, the GCSF 3' UTS was removed from pXAG-4 as an XbaI-SmaI fragment and the ends of pXAG-4 were made blunt. The hGH 3' UTS was removed from pXGH5 (Selden et al., Mol. Cellular Biol. 6:3173-3179,



- 19 -

1986) as a 0.6 kb SmaI-EcoRI fragment. After blunt-ending this fragment, it was ligated into pXAG-4 immediately after the blunt-ended XbaI site of pXAG-4. This intermediate was called pXAG-7. The TKneo fragment  
5 was removed from this plasmid as a HindIII-ClaI fragment and the ends of the plasmid were blunted by "filling-in" with the Klenow fragment of DNA polymerase I. A neomycin resistance gene driven by the SV40 early promoter was  
10 ligated in as a blunted ClaI-BsmBI fragment from a digest of pcDNeo (Chen et al., Mol. Cellular Biol. 7:2745-2752, 1987), placing the neo transcription unit in the same orientation as the  $\alpha$ -gal A transcription unit. This intermediate was called pXAG-13.

To complete pXAG-16, which has the 26 amino acid  
15 hGH signal peptide coding sequence and first intron of the hGH gene, a 2.0 kb EcoRI-BamHI fragment of pXAG-13 was first removed. This fragment included the  $\alpha$ -gal A cDNA and the hGH 3' UTS. This large fragment was replaced with 3 fragments. The first fragment consisted  
20 of a 0.3 kb PCR product of pXGH5, which contains the hGH signal peptide coding sequence and includes the hGH first intron sequence, from a synthetic BamHI site located just upstream of the Kozak consensus sequence to the end of the hGH signal peptide coding sequence. The following  
25 oligonucleotides were used to amplify this fragment (Fragment 1): 5'-TTTTGGATCCACCATGGCTA-3' (Oligo HGH101; SEQ ID NO:5) and 5'-TTTTGCCGGCACTGCCCTCTTGAA-3' (Oligo HGH102; SEQ ID NO:6). The second fragment consisted of a 0.27 kb PCR product containing sequences  
30 corresponding to the start of the cDNA encoding the 398 amino acid  $\alpha$ -gal A enzyme (i.e., lacking the  $\alpha$ -gal A signal peptide) to the NheI site. The following oligonucleotides were used to amplify this fragment (Fragment 2): 5'-TTTTCAGCTGGACAATGGATTGGC-3' (Oligo AG10;  
35 SEQ ID NO:7) and 5'-TTTTGCTAGCTGGCGAATCC-3' (Oligo AG11;

- 20 -

SEQ ID NO:8). The third fragment consisted of the NheI-EcoRI fragment of pXAG-7 containing the remaining  $\alpha$ -gal A sequence as well as the hGH 3' UTS (Fragment 3).

Fragment 1 (digested with BamHI and NaeI),  
5 Fragment 2 (digested with PvuII and NheI), and Fragment 3 were mixed with the 6.5 kb BamHI-EcoRI fragment of pXAG-13 containing the neo gene and the CMV IE promoter and ligated together to generate plasmid pXAG-16 (Figure 4).

#### B. Construction of the $\alpha$ -Gal A

##### 10 Expression Plasmid pXAG-28

The human collagen I $\alpha$ 2 promoter was isolated for use in the  $\alpha$ -gal A expression construct pXAG-28 as follows. A 408 bp PCR fragment of human genomic DNA containing part of the human collagen I $\alpha$ 2 promoter was  
15 isolated using the following oligonucleotides:

5'-TTTGGATCCGTGTCCCATAGTGTTCCTCAA-3' (Oligo 72;  
SEQ ID NO:9) and 5'-TTTGGATCCGTCAGTCGTGGCCAGTACC-3'  
(Oligo 73; SEQ ID NO:10).

This fragment was used to screen a human leukocyte  
20 library in EMBL3 (Clontech Inc., Palo Alto, CA). One positive clone (phage 7H) containing a 3.8 kb EcoRI fragment was isolated and cloned into pBSIISK+ (Stratagene Inc., La Jolla, CA) at the EcoRI site (creating pBS/7H.2). An AvrII site was introduced in  
25 pBSIISK+ by digesting with SpeI, which cleaves within the pBSIISK+ polylinker, "filling-in" with the Klenow fragment of DNA polymerase I, and inserting the oligonucleotide 5'-CTAGTCCTAGGA-3' (SEQ ID NO:11). This variant of pBSIISK+ was digested with BamHI and AvrII and  
30 ligated to the 121 bp BamHI-AvrII fragment of the original 408 bp collagen I $\alpha$ 2 promoter PCR fragment described above, creating pBS/121COL.6.

The plasmid pBS/121COL.6 was digested with XbaI, which cleaves within the pBSIISK+ polylinker sequence,  
35 "filled-in" with the Klenow fragment of DNA polymerase I,

- 21 -

and digested with AvrII. The 3.8 kb BamHI-AvrII fragment of pBS/7H.2 was isolated and the BamHI site made blunt-ended by treatment with Klenow enzyme. The fragment was then digested with AvrII and ligated to the  
5 AvrII-digested vector, thus creating the collagen promoter plasmid pBS/121bpCOL7H.18.

Next the collagen promoter was fused to the 5' UTS of the human  $\beta$ -actin gene, which contains the first intron of the human  $\beta$ -actin gene. To isolate this  
10 sequence, a 2 kb PCR fragment was isolated from human genomic DNA using the following oligonucleotides:

5'-TTTGTAGCACAGAGCCTCGCCT-3' (Oligo BA1; SEQ ID NO:12)

and

5'-TTTGGATCCGGTGAGCTGCGAGAATAGCC-3' (Oligo BA2;

15 SEQ ID NO:13).

This fragment was digested with BamHI and BsiHKA1 to release a 0.8 kb fragment containing the  $\beta$ -actin 5' UTS and intron. A 3.6 kb SalI-SrfI fragment was then isolated from the collagen promoter plasmid  
20 pBS/121bpCOL7H.18 as follows. pBS/121bpCOL7H.18 was partially digested with BamHI (the BamHI site lies at the 5' end of the collagen I $\alpha$ 2 promoter fragment), made blunt-ended by treatment with the Klenow fragment, and ligated to a SalI linker (5'-GGTCGACC-3'), thereby  
25 placing a SalI site upstream of the collagen I $\alpha$ 2 promoter. This plasmid was then digested with SalI and SrfI (the SrfI site lies 110 bp upstream of the collagen I $\alpha$ 2 promoter CAP site), and the 3.6 kb fragment was isolated. The 0.8 and 3.6 kb fragments were combined with SalI -  
30 and BamHI - digested pBSIISK- (Stratagene Inc., La Jolla, CA), and a fragment composed of the following four oligonucleotides annealed together (forming a fragment with a blunt end and a BsiHKA1 end):  
5'-GGGCCCCCAGCCCCAGCCCTCCCATTTGGTGGAGGCCCTTTTGGAGGCACCCTAG  
35 GGCCAGGAAACTTTTGCCGTAT-3' (Oligo COL-1; SEQ ID NO:14),

- 22 -

5'-AAATAGGGCAGATCCGGGCTTTATTATTTTAGCACCACGGCCGCCGAGAC  
 CGCGTCCGCCCCGCGAGCA-3' (Oligo COL-2; SEQ ID NO:15),  
 5'-TGCCCTATTTATACGGCAAAAGTTTCCTGGCCCTAGGGTGCCTCCAAAAGGGC  
 CTCCACCAATGGGAGGGCTGGGGCTGGGGGCCC-3' (Oligo COL-3;  
 5 SEQ ID NO:16), and 5'-CGCGGGGCGGACGCGGTCTCGGCGGCCGTGGTGCT  
 AAAATAATAAGCCCGGATC-3' (Oligo COL-4; SEQ ID NO:17).  
 These four oligonucleotides, when annealed, correspond to  
 the region beginning at the SrfI site of the collagen  
 promoter and continuing through the BsiHKAI site of the  
 10  $\beta$ -actin promoter. The resulting plasmid was designated  
 pCOL/ $\beta$ -actin.

To complete the construction of pXAG-28, the  
 Sall-BamHI fragment of pCOL/ $\beta$ -actin, containing the  
 collagen I $\alpha$ 2 promoter and  $\beta$ -actin 5' UTS, was isolated.  
 15 This fragment was ligated to two fragments from pXAG-16  
 (see Example 1A and Fig. 4): (1) the 6.0 kb BamHI  
 fragment (containing the neo gene, plasmid backbone, the  
 cDNA encoding the 398 amino acid  $\alpha$ -gal A enzyme, and the  
 hGH 3' UTS); and (2) the 0.3 kb BamHI-XhoI fragment  
 20 (which contains the SV40 poly A sequence from pCDneo).  
 pXAG-28 contains the human collagen I $\alpha$ 2 promoter fused to  
 the human  $\beta$ -actin 5' UTS, the hGH signal peptide (which  
 is interrupted by the hGH first intron), the cDNA  
 encoding the  $\alpha$ -gal A enzyme, and the hGH 3' UTS. A map  
 25 of the completed expression construct pXAG-28 is shown in  
 Fig. 5.

### C. Transfection and Selection of Fibroblasts Electroporated with $\alpha$ -Gal A Expression Plasmids

In order to express  $\alpha$ -gal A in fibroblasts,  
 30 secondary fibroblasts were cultured and transfected  
 according to published procedures (Selden et al.,  
 WO 93/09222).

The plasmids pXAG-13, pXAG-16 and pXAG-28 were  
 transfected by electroporation into human foreskin  
 35 fibroblasts to generate stably transfected clonal cell

- 23 -

strains, and the resulting  $\alpha$ -gal A expression levels were monitored as described in Example ID. Secretion of  $\alpha$ -gal A by normal foreskin fibroblasts is in the range of 2-10 units/ $10^6$  cells/24 hours. In contrast, the

5 transfected fibroblasts displayed mean expression levels as shown in Table 1:

Table 1: Mean  $\alpha$ -gal A expression levels (+/- standard deviation)

10	pXAG-13:	420 +/- 344 U/ $10^6$ cells/day N=26 clonal strains (range 3 - 1133 U/ $10^6$ cells/day)
15	pXAG-16:	2,051 +/- 1253 U/ $10^6$ cells/day N=24 clonal strains (range 422 - 5200 U/ $10^6$ cells/day)
	pXAG-28:	141 +/- 131 U/ $10^6$ cells/day N=38 clonal strains (range 20 - 616 U/ $10^6$ cells/day)

These data show that all three expression

20 constructs are capable of increasing  $\alpha$ -gal A expression many times that of nontransfected fibroblasts. Expression by fibroblasts stably transfected with pXAG-13, which encodes  $\alpha$ -gal A linked to the  $\alpha$ -gal A signal peptide, was substantially lower than expression by

25 fibroblasts transfected with pXAG-16, which differs only in that the signal peptide is the hGH signal peptide, the coding sequence of which is interrupted by the first intron of the hGH gene.

- 24 -

Each time the transfected cells were passaged, the secreted  $\alpha$ -gal A activity was determined, the cells were counted, and the cell density was calculated. Based on the number of cells harvested and the time allowed for secretion of  $\alpha$ -gal A, the specific expression rate of  $\alpha$ -gal A was determined and is reported in Tables 2 and 3 as secreted units (of  $\alpha$ -gal A) per  $10^6$  cells per 24 hour period. Cell strains desirable for gene therapy or for use in generation of material for purification of  $\alpha$ -gal A should display stable growth and expression over several passages. Data from the cell strains shown in Tables 2 and 3, which were stably transfected with the  $\alpha$ -gal A expression construct pXAG-16, illustrate the fact that  $\alpha$ -gal A expression is stably maintained during serial passage.

Table 2: Growth and Expression of BRS-11 Cells  
Containing the  $\alpha$ -Gal A Expression Construct  
pXAG-16.

BRS-11		
Passage	Expression (units/ $10^6$ cells/24 hr)	Cell Density (cells/cm <sup>2</sup> )
13	2601	$4.80 \times 10^4$
14	1616	$4.40 \times 10^4$
15	3595	$4.40 \times 10^4$

- 25 -

Table 3: Growth and Expression of HF503-242 Cells  
Containing the  $\alpha$ -Gal A Expression Construct  
pXAG-16.

HF503-242		
Passage	Expression (units/ $10^6$ cells/24hr)	Cell Density (cells/ $\text{cm}^2$ )
5	4069	$2.80 \times 10^4$
6	7585	$3.55 \times 10^4$
7	5034	$2.48 \times 10^4$

D. Quantification of  $\alpha$ -Gal A Expression

The activity of  $\alpha$ -gal A activity was measured using the water-soluble substrate 4-methylumbelliferyl- $\alpha$ -D-galactopyranoside (4-MUF-gal; Research Products, Inc.) by a modification of the protocol described by Ioannou et al. (J. Cell Biol. 119:1137-1150, 1992). The substrate was dissolved in substrate buffer (0.1 M citrate-phosphate, pH 4.6) to a concentration of 1.69 mg/ml (5 mM). Typically, 10  $\mu$ l of culture supernatant was added to 75  $\mu$ l of the substrate solution. The tubes were covered and allowed to incubate in a 37°C waterbath for 60 minutes. At the end of the incubation period, 2 ml of glycine-carbonate buffer (130 mM glycine, 83 mM sodium carbonate, at pH 10.6), were used to stop the reaction. The relative fluorescence of each sample was measured using a model TK0100 fluorometer (Hoefer Scientific Instruments) which has a fixed excitation wavelength of 365 nm and detects a fixed emission wavelength of 460 nm. The

- 26 -

readings of the samples were compared to standards prepared from a 1  $\mu$ M stock of methylumbelliferone (Sigma Chemical Co.), and the amount of hydrolyzed substrate was calculated. The activity of  $\alpha$ -gal A is expressed in 5 units; one unit of  $\alpha$ -gal A activity is equivalent to one nanomole of substrate hydrolyzed per hour at 37°C. Cell expression data were generally expressed as units of  $\alpha$ -gal A activity secreted/ $10^6$  cells/24 hours. This assay was also used to measure the amount of  $\alpha$ -gal activity in 10 cell lysates and in samples from various  $\alpha$ -gal purification steps, as discussed below.

Example II. Purification of  $\alpha$ -Gal A from the Conditioned Medium of Stably Transfected Human Cell Strains

Examples IIA-IIE illustrate that  $\alpha$ -gal A may be 15 purified to near-homogeneity from the conditioned medium of cultured human cell strains that have been stably transfected to produce the enzyme.

A. Use of Butyl Sepharose<sup>®</sup> Chromatography as a First Step in the Purification of  $\alpha$ -Gal A

20 Cold conditioned medium (1.34 liters) was clarified by centrifugation and filtered through a 0.45  $\mu$ m cellulose acetate filter using glass fiber prefilters. While stirring, the pH of the cold, filtered medium was adjusted to 5.6 by the dropwise addition of 25 1 N HCl, and ammonium sulfate was added to a final concentration of 0.66 M by the dropwise addition of a stock solution (room temperature) of 3.9 M ultrapure ammonium sulfate. The medium was stirred for an additional 5 minutes at 4°C, filtered as before, and 30 applied to a Butyl Sepharose<sup>®</sup> 4 Fast Flow column (81 ml column volume, 2.5 x 16.5 cm; Pharmacia, Uppsala, Sweden) that had been equilibrated in 10 mM MES-Tris, pH 5.6, containing 0.66 M ammonium sulfate (buffer A). The chromatography was performed at 4°C on a Gradi-Frac™



- 27 -

System (Pharmacia, Uppsala, Sweden) equipped with in-line UV (280 nm) and conductivity monitors for assessing total protein and salt concentration, respectively. After sample application at a flow rate of 10 ml/min, the

5 column was washed with 10 column volumes of buffer A. The  $\alpha$ -gal A was eluted from the Butyl Sepharose® column with a 14 column volume linear gradient from buffer A (containing ammonium sulfate) to 10 mM MES-Tris, pH 5.6 (no ammonium sulfate). Fractions were assayed for

10  $\alpha$ -gal A activity by the 4-MUF-gal assay, and those containing appreciable enzyme activity were pooled. As seen in Fig. 6 and the purification summary (Table 3), this step removes approximately 99% of the contaminating protein (pre-column sample=8.14 g total protein; post-

15 column sample=0.0638 g total protein).

- 28 -

Table 4: Purification of  $\alpha$ -Gal A from the Conditioned Medium of Stably Transfected Human Fibroblasts

Purification Step	Volume (ml)	$\alpha$ -Gal A Activity ( $\times 10^6$ Units)	Total Protein (mg)	Specific Activity ( $\times 10^6$ Units/mg)	Fold Purification (Cumulative)	Percent Recovery
5 Culture supernatant	1340	14.6	8140	0.0018	=1	=100
10 Butyl Sepharose <sup>®</sup>	417	14.1	63.8	0.221	123	96.6
Heparin Sepharose <sup>®</sup>	134	12.1	14.6	0.829	436	82.9
15 Hydroxyapatite	47	9.73	4.46	2.18	1220	66.6
Q Sepharose <sup>®</sup>	31.5	8.91	3.31	2.69	1503	61.0
20 Superdex <sup>®</sup> 200	10	8.58	2.93	2.92	1634	59.0

B. Use of Heparin Sepharose<sup>®</sup> Chromatography as a Step for Purification of  $\alpha$ -Gal A

The Butyl Sepharose<sup>®</sup> column peak fractions were dialyzed at 4°C against (4 liters) of 10 mM MES-Tris, pH 5.6 (changed once). The conductivity of the dialysate was adjusted to 1.0 mMH<sub>2</sub>O at 4°C by addition of H<sub>2</sub>O or NaCl as necessary. Afterward, the sample was applied to a column of Heparin Sepharose<sup>®</sup> 6 Fast Flow (Pharmacia, Uppsala, Sweden; 29 ml column volume, 2.5 x 6 cm) that had been equilibrated in 10 mM MES-Tris, pH 5.6, containing 9 mM NaCl (buffer B). This was done at 4°C at a flow rate of 10 ml/min. In-line UV (280 nm) and

- 29 -

conductivity monitors measured total protein and salt concentration. After the sample was applied, the column was washed with 10 column volumes of buffer B followed by a 3 column volume linear gradient to 8% buffer C/92%  
5 buffer B (where buffer C is 10 mM MES-Tris, pH 5.6, containing 250 mM NaCl) and a 10 column volume wash with 8% buffer C. This was followed by elution of  $\alpha$ -gal A with a 1.5 column volume linear gradient to 29% buffer C and a subsequent 10 column volume linear gradient to  
10 35% buffer C. Fractions were assayed for  $\alpha$ -gal A activity, and those containing appreciable activity were pooled.

C. Use of Hydroxyapatite  
Chromatography as a Step for  
15 Purification of  $\alpha$ -Gal A

The heparin pool was filtered and applied directly to a column of Ceramic Hydroxyapatite HC (40  $\mu$ m; American International Chemical, Natick, MA; 12 ml column volume, 1.5 x 6.8 cm) that had been equilibrated in 1 mM  
20 sodium phosphate, pH 6.0 (buffer D). The chromatography was performed at room temperature on a hybrid Gradi-Frac<sup>®</sup>/FPLC<sup>®</sup> System (Pharmacia, Uppsala, Sweden) equipped with in-line UV (280 nm) and conductivity monitors. After the sample was applied (5 ml/min), the column was  
25 washed with 10 column volumes of buffer D. The  $\alpha$ -gal A was eluted with a 7 column volume linear gradient to 42% buffer E/58% buffer D (where buffer E is 250 mM sodium phosphate, pH 6.0) followed by a 10 column volume gradient to 52% buffer E. Fractions were assayed for  $\alpha$ -  
30 gal A activity, and the fractions containing appreciable activity were pooled.

- 30 -

D. Use of Q Sepharose® Anion  
Exchange Chromatography as a Step  
for Purification of  $\alpha$ -Gal A

The hydroxyapatite pool was diluted  
5 approximately 1.5 fold with H<sub>2</sub>O to a final conductivity  
of 3.4-3.6 mMHO at room temperature. After filtering,  
the sample was applied to a column of Q Sepharose® HP  
(Pharmacia, Uppsala, Sweden; 5.1 ml column volume,  
1.5 x 2.9 cm) equilibrated in 10% buffer G/90% buffer F,  
10 where buffer F is 25 M sodium phosphate, pH 6.0, and  
buffer G is 25 mM sodium phosphate, pH 6.0, 250 mM NaCl.  
The chromatography was performed at room temperature on  
the Gradi-Frac™/FPLC® hybrid system (Pharmacia, Uppsala,  
Sweden), and total protein and salt concentrations were  
15 monitored by the in-line monitors. The sample was  
applied at a flow rate of 5 ml/min, then the following  
steps were performed: (1) a 5 column volume wash at  
10% buffer G, (2) a 7 column volume wash at 12% buffer G,  
(3) a 3 column volume linear gradient to 50% buffer G,  
20 (4) a 10 column volume linear gradient to 53% buffer G,  
(5) a 3 column volume gradient to 100% buffer G, and  
(6) a 10 column volume wash at 100% buffer G. The  $\alpha$ -  
gal A eluted primarily during steps 3 and 4. Fractions  
containing appreciable activity were pooled (the  
25 "Q pool").

E. Use of Superdex®-200 Gel  
Filtration Chromatography as a Step  
for Purification of  $\alpha$ -Gal A

The Q pool was concentrated approximately 5-  
30 fold using Centriprep®-10 centrifugal concentrator units  
(Amicon, Beverly, MA), and applied to a column of  
Superdex® 200 (Pharmacia, Uppsala, Sweden; 189 ml column  
volume, 1.6 x 94 cm). The column was equilibrated and  
eluted with 25 mM sodium phosphate, pH 6.0, containing  
35 150 mM NaCl. The chromatography was performed on an

- 31 -

FPLC® system (Pharmacia, Uppsala, Sweden) at room temperature using an in-line UV monitor (280 nm) to follow elution of the protein. The volume of the sample applied to the column was  $\leq 2$  ml, the flow rate was 0.5 ml/min, and the fraction size was 2 ml. Multiple column runs were performed; fractions were assayed for  $\alpha$ -gal A activity and fractions containing appreciable activity were pooled.

The pooled fractions from the Superdex® 200 column were concentrated using Centriprep-10 units, aliquoted, snap-frozen, and stored at  $-80^{\circ}\text{C}$  for short periods of time. A summary of this example of  $\alpha$ -gal A purification is shown in Table 3. The final yield of  $\alpha$ -gal A was 59% of the starting material activity, and the specific activity of the purified product was  $2.92 \times 10^6$  units/mg protein. The resulting product showed a high level of purity after electrophoresis under reducing conditions on a 4-15% SDS-polyacrylamide gel, which was subsequently silver-stained.

20 Example III. Formulation and Storage of Purified  $\alpha$ -Gal A

Highly purified  $\alpha$ -gal A is not stable for extended periods of time when stored as a dilute solution of purified protein ( $\leq 1$  mg protein/ml). Therefore, a formulation was devised to improve stability during prolonged storage, i.e. storage lasting several weeks to at least several months. The purified enzyme was concentrated to at least 1 mg/ml using a centrifugal concentrator (in enzyme buffer consisting of 25 mM sodium phosphate (pH 6.0) and 150 mM NaCl). Human serum albumin (HSA; Buminate®, Baxter-Hyland) was added to a final concentration of 2.5 mg/ml. The protein solution was then sterile filtered using a  $0.2 \mu\text{m}$  cellulose acetate filter (Schleicher and Schuell) attached to a syringe. The  $\alpha$ -gal A solution was dispensed into sterile, pyrogen-

- 32 -

free glass vials, sealed with a Teflon cap, snap-frozen, and stored at  $-20^{\circ}\text{C}$ .

Stability of the  $\alpha$ -gal A activity was evaluated over a three month period using the 4-MUF-gal assay. The data presented in Table 5 demonstrate that there was no loss of enzyme activity over the test period. The acidic pH of the formulation ( $< 6.5$ ) is critical to the stability of the highly purified enzyme.

Table 5: Stability of Formulated  $\alpha$ -Gal A at  $-20^{\circ}\text{C}$

10	Sample	Specific Activity (Units/mg total protein)
	time 0	$2.24 \times 10^6 \pm 0.33 \times 10^6$
	week 1	$2.40 \times 10^6 \pm 0.25 \times 10^6$
	week 2	$2.42 \times 10^6 \pm 0.21 \times 10^6$
	week 3	$2.37 \times 10^6 \pm 0.05 \times 10^6$
15	month 1	$2.39 \times 10^6 \pm 0.16 \times 10^6$
	month 2	$2.31 \times 10^6 \pm 0.26 \times 10^6$
	month 3	$2.29 \times 10^6 \pm 0.17 \times 10^6$

- 33 -

Example IV.  $\alpha$ -Gal A Produced by Human Cell Strains is Suitable for Treatment of  $\alpha$ -Gal A Deficiency

The structural and functional properties of purified human  $\alpha$ -gal A prepared in accordance with the invention were investigated in order to demonstrate that the DNA molecules described herein and the corresponding expressed glycoproteins produced by transfected human cell strains can be used in gene or enzyme replacement therapies, respectively.

10           A. Size of  $\alpha$ -Gal A Produced by Stably Transfected Human Cells in Culture

The molecular mass of  $\alpha$ -gal A was estimated by MALDI-TOF mass spectrometry. These results demonstrate that the molecular mass of the dimer is 102,353 Da, while that of the monomer is 51,002 Da. The expected molecular mass of the monomer, based on amino acid composition, is 45,400 Da. Therefore, it can be inferred that the carbohydrate content of the enzyme accounts for up to 5,600 Da of the molecular weight.

20           The results of standard amino acid analysis performed on the purified protein are consistent with the conclusion that the protein produced by transfected human cells is identical to the protein purified from human tissues at the amino acid level.

25           B. N-Terminal Processing of  $\alpha$ -Gal A Produced by Stably Transfected Human Cells

The human  $\alpha$ -gal A cDNA nucleotide sequence encodes 429 amino acids. The 31 N-terminal amino acids constitute a signal peptide sequence, which is cleaved as the nascent protein transits the endoplasmic reticulum membrane (LeDonne et al., Arch. Biochem. Biophys. 224:186, 1983; Lemansky et al., J. Biol. Chem. 262:2062, 1987). In order to confirm that  $\alpha$ -gal A is properly processed when associated with an heterologous signal peptide sequence (for example, the human growth hormone

35

- 34 -

signal sequence) and expressed in transfected human fibroblasts, ten N-terminal amino acids of the secreted protein were microsequenced. Samples were electrophoresed by SDS-PAGE and transferred to ProBlott® (ABI, Foster City, CA) using a 10 mM CAPS (pH 11.0), 10% methanol buffer system. The protein on the ProBlott® was visualized by Coomassie staining, and an appropriately sized (50 kDa) band was excised. N-terminal sequence was obtained using an Applied Biosystems pulse-liquid phase amino acid sequenator that performs automated Edman degradation. The N-terminal sequence obtained, LDNGLARTPT (SEQ ID NO:28), is consistent with proper cleavage of the signal peptide and matches the N-terminal sequence predicted for the secreted protein.

C. C-Terminal Amino Acid of  $\alpha$ -Gal A  
Produced by Stably Transfected Human Cells

The C-terminal amino acid residue of secreted  $\alpha$ -gal A produced in accordance with the invention was identified using an automated Hewlett Packard C-terminal sequencer. The results indicated a leucine residue at the C-terminus, which agrees with the C-terminal amino acid predicted by the DNA sequence.

D. Carbohydrate Modification of  $\alpha$ -Gal A  
Produced by Stably Transfected Human Cells

The glycosylation pattern of  $\alpha$ -gal A produced in accordance with the invention was also evaluated. Proper glycosylation is important for optimal *in vivo* activity of  $\alpha$ -gal A;  $\alpha$ -gal A expressed in non-glycosylating systems is inactive or unstable (Hantzopolous et al., Gene 57:159, 1987). Glycosylation is also important for the internalization of  $\alpha$ -gal A into the desired target cells, and affects the circulating half-life of the enzyme *in vivo*. On each subunit of  $\alpha$ -gal A there are four sites available for addition of



- 35 -

asparagine-linked carbohydrate chains, of which only three are occupied (Desnick et al., In The Metabolic and Molecular Bases of Inherited Disease, pp 2741-2780, McGraw Hill, New York, 1995).

5           A sample of  $\alpha$ -gal A produced by stably transfected cells was treated with neuraminidase, which is isolated from *A. urafaciens*, (Boehringer-Mannheim, Indianapolis, IN) to remove sialic acid. This reaction was performed by treating 5  $\mu$ g of  $\alpha$ -gal A overnight with  
10 10 mU of neuraminidase at room temperature in a total volume of 10  $\mu$ l of acetate buffered saline (ABS, 20 mM sodium acetate, pH. 5.2, 150 mM NaCl).

Purified  $\alpha$ -gal A produced by stably transfected cells was also dephosphorylated using alkaline  
15 phosphatase (calf intestinal alkaline phosphatase, Boehringer-Mannheim, Indianapolis, IN), by treating 5  $\mu$ g of  $\alpha$ -gal A overnight at room temperature with 15 U of alkaline phosphatase in ABS (pH raised to 7.5 with 1 M Tris).

20           The samples were analyzed by Western blot with an  $\alpha$ -gal A-specific antibody. The antibody used was a rabbit polyclonal anti-peptide antibody, which was produced using a peptide representing amino acids 68-81 of  $\alpha$ -gal A as an immunogen. Following transfer of the  
25 protein to PVDF (Millipore, Bedford, MA), the membrane was probed with a 1:2000 dilution of the anti-serum in 2.5% blotto (non-fat dry milk in 20 mM Tris-HCl, pH 7.5, 0.05% Tween-20). This was followed by detection with goat anti-rabbit IgG conjugated to horseradish peroxidase  
30 (Organon Teknika/Cappel, Durham, NC; 1:5000 dilution) and reagents of the ECL chemiluminescence kit (Amersham, Arlington Heights, IN).

Treatment of  $\alpha$ -gal A with neuraminidase results in a slight shift in molecular mass (approximately 1500-  
35 2000 Da or 4-6 sialic acids/monomer), suggesting that

- 36 -

there is extensive modification of  $\alpha$ -gal A with sialic acid. For reference, the plasma form of  $\alpha$ -gal A has 5-6 sialic acid residues per monomer, and the placental form has 0.5-1.0 sialic acid residues per monomer (Bishop et al., J. Biol. Chem. 256:1307, 1981).

Another method used to examine the sialic acid and mannose-6-phosphate modifications of  $\alpha$ -gal A was isoelectric focusing (IEF), where the samples are separated on the basis of their isoelectric point (pI) or net charge. Thus, removal of charged residues such as sialic acid or phosphate from  $\alpha$ -gal A would be expected to alter the mobility of the protein in the IEF system.

To perform the IEF experiment, samples of  $\alpha$ -gal A produced in accordance with the invention were treated with neuraminidase and alkaline phosphatase, mixed 1:1 with 2X Novex sample buffer (with 8 M urea, pH 3.0-7.0), and loaded onto a 6 M urea IEF gel (5.5% polyacrylamide) made using Pharmalyte® (Pharmacia, Uppsala, Sweden; pH 3.0-6.5; Pharmalyte® 4-6.5 and 2.5-5.5, 0.25 ml each per gel). Isoelectric point standards (Bio-Rad) were also included. Following electrophoresis, the gel was transferred to PVDF, and Western blot analysis performed as described above.

The  $\alpha$ -gal A produced by stably transfected human fibroblasts consisted of three major isoforms with a pI range of approximately 4.4-4.65. These values are similar to the pIs of the plasma and splenic forms of  $\alpha$ -gal A (Bishop et al., J. Biol. Chem. 256:1307, 1981). Neuraminidase treatment of the enzyme increased the pI of all three isoforms, indicating that all were modified to some extent by sialic acid. These data suggest that the  $\alpha$ -gal A produced by stably transfected human cells should have a desirable plasma half-life, indicating that this material is well suited for pharmacologic use. Further, treatment of neuraminidase-treated  $\alpha$ -gal A with alkaline

- 37 -

phosphatase further increased the pI of a portion of the protein to approximately 5.0-5.1, indicating that the enzyme bears one or more mannose-6-phosphate residues. This modification is significant in that it is required for efficient internalization of  $\alpha$ -gal A by the target cells.

#### E. Specific Activity of $\alpha$ -gal A

##### Purified from Stably Transfected Fibroblasts

The potency or specific activity of purified  $\alpha$ -gal A is calculated by measuring both the catalytic activity of the enzyme (with the 4-MUF-gal assay), and the protein concentration. The protein concentration can be determined by any standard method, such as with the BCA system (Pierce), or by measuring the absorbance at 280 nm and using the mg/ml extinction coefficient of 2.3 (determined from amino acid analysis) to calculate the value. Using these techniques, the specific activity of  $\alpha$ -gal A purified from the conditioned medium of transfected human fibroblasts is  $2.2-2.9 \times 10^6$  units/mg of protein, which is comparable to the specific activity of  $\alpha$ -gal A that is purified from human tissues (Bishop et al., J. Biol. Chem. 256:1301, 1981).

#### F. Mannose or Mannose-6-Phosphate Mediated Internalization of $\alpha$ -Gal A

In order for the  $\alpha$ -gal A produced by stably transfected cells to be an effective therapeutic agent for  $\alpha$ -gal A deficiencies, the enzyme must be internalized by the affected cells.  $\alpha$ -Gal A is not active at physiological pH levels, and is unlikely to be effective in the blood or interstitial fluids. It metabolizes accumulated lipid substrates optimally only when internalized in the acidic environment of the lysosome. This internalization is mediated by the binding of  $\alpha$ -gal A to mannose-6-phosphate (M6P) receptors, which are expressed on the cell surface and deliver the enzyme to

- 38 -

the lysosome via the endocytic pathway. The M6P receptor is ubiquitously expressed; most somatic cells express it to some extent. The mannose receptor, which is specific for exposed mannose residues on glycoproteins, is less prevalent. The latter receptors are generally found only on macrophage and macrophage-like cells, and provide an additional means of  $\alpha$ -gal A entry into these cell types.

In order to demonstrate M6P-mediated internalization of  $\alpha$ -gal A, skin fibroblasts from a Fabry disease patient (NIGMS Human Genetic Mutant Cell Repository) were cultured overnight in the presence of increasing concentrations of purified  $\alpha$ -gal A of the invention. Some of the samples contained 5 mM soluble M6P, which competitively inhibits binding to, and as a result, internalization by, the mannose-6 phosphate receptor. Other samples contained 30  $\mu$ g/ml mannan, which inhibits binding to, and as a result, internalization by, the mannose receptor. Following incubation, the cells were washed and harvested by scraping into lysis buffer (10 mM Tris, pH 7.2, 100 mM NaCl, 5 mM EDTA, 2 mM Pefabloc™ (Boehringer-Mannheim, Indianapolis, IN) and 1% NP-40). The lysed samples were then assayed for protein concentration and  $\alpha$ -gal A activity. The results are expressed as units of  $\alpha$ -gal A activity/mg cell protein. The Fabry cells internalized  $\alpha$ -gal A in a dose-dependent manner (Fig. 7). This internalization was inhibited by mannose-6 phosphate, but there was no inhibition with mannan. Therefore, internalization of  $\alpha$ -gal A in Fabry fibroblasts is mediated by the mannose-6 phosphate receptor, but not by the mannose receptor.

$\alpha$ -gal A is also internalized *in vitro* by endothelial cells, important target cells for the treatment of Fabry disease. Human umbilical vein endothelial cells (HUVECs) were cultured overnight with 7500 units of  $\alpha$ -gal A; some of the wells contained M6P.

- 39 -

After the incubation period, cells were harvested and assayed for  $\alpha$ -gal A as described above. The cells incubated with  $\alpha$ -gal A only had enzyme levels almost 10-fold above those of control (no incubation with  $\alpha$ -gal A) cells. M6P inhibited the intracellular accumulation of  $\alpha$ -gal A, suggesting that the internalization of  $\alpha$ -gal A by HUVECs is mediated by the M6P receptor. Thus, the human  $\alpha$ -gal A of the invention is internalized by clinically relevant cells.

10 Few cultured human cell lines are known to express the mannose receptor. However, a mouse macrophage-like cell line (J774.E) which bears mannose receptors but few if any mannose 6-phosphate receptors can be used to determine whether purified  $\alpha$ -gal A of the  
15 invention is internalized via the mannose receptor (Diment et al., J. Leukocyte Biol. 42:485-490, 1987). J774.E cells were cultured overnight in the presence of 10,000 units/ml  $\alpha$ -gal A. Selected samples also contained 2 mM M6P, and others contained 100  $\mu$ g/ml mannan. The  
20 cells were washed and harvested as described above, and the total protein and  $\alpha$ -gal A activity of each sample was determined. The results are shown in Table 5. M6P does not inhibit the uptake of  $\alpha$ -gal A by these cells, while mannan decreases the accumulated  $\alpha$ -gal A levels by 75%.  
25 Thus, the  $\alpha$ -gal A of the invention may be internalized by the mannose receptor in cell types that express this particular cell surface receptor.

- 40 -

Table 6. Internalization of  $\alpha$ -Gal A by J774.E Cells.

$\alpha$ -Gal A Activity (units/mg total protein)				
	No additions	+ $\alpha$ -gal A	+ $\alpha$ -gal A, + M6P	+ $\alpha$ -gal A, + mannan
J774.E	409 $\pm$ 25	6444 $\pm$ 554	6297 $\pm$ 674	1654 $\pm$ 323

[mannan] = 100  $\mu$ g/ml5 [M6P] = 2 mM or 660  $\mu$ g/ml

These experiments demonstrate that the  $\alpha$ -gal A produced by stably transfected human cells may be internalized by cells via the mannose or mannose-6-phosphate receptor.

10 G. Correction of Fabry Fibroblasts by Human Fibroblasts Expressing  $\alpha$ -Gal A

For gene therapy, an implant of autologous cells producing  $\alpha$ -gal A must produce the enzyme in a form modified appropriately to "correct" the  $\alpha$ -gal A

15 deficiency in target cells. To assess the effect of  $\alpha$ -gal A production by transfected human fibroblasts on Fabry cells, fibroblasts harvested from Fabry disease patients (NIGMS Human Genetics Mutant Cell Repository) were co-cultured with an  $\alpha$ -gal A-producing cell strain

20 (BRS-11) in Transwells<sup>®</sup> (Costar, Cambridge, MA). The experimental scheme is depicted in Fig. 8. Fabry cells were cultured in 12-well tissue culture dishes, some of which contained inserts (Transwells<sup>®</sup>, 0.4  $\mu$ m pore size) having a surface on which cells can be grown. The growth

25 matrix of the insert is porous and allows macromolecules to pass from the upper to the lower milieu. One set of inserts contained normal human foreskin (HF) fibroblasts,

- 41 -

which secrete minimal levels of  $\alpha$ -gal A, while another set contained the stably transfected human fibroblast strain, BRS-11, which secretes large amounts of  $\alpha$ -gal A. In the wells co-cultured with  $\alpha$ -gal A-producing cells,  $\alpha$ -gal A can enter the medium bathing the Fabry cells, and potentially be internalized by the Fabry cells.

The data in Table 7 show that Fabry cells internalized the secreted  $\alpha$ -gal A. The intracellular levels of  $\alpha$ -gal A were monitored for three days. Those cells cultured alone (no insert) or in the presence of non-transfected foreskin fibroblasts (HF insert) had very low intracellular levels of  $\alpha$ -gal A activity. The Fabry cells cultured with the  $\alpha$ -gal A-producing (BRS-11 insert) cells, however, exhibited enzyme levels similar to those of normal cells by the end of Day 2 (normal fibroblasts have 25-80 units  $\alpha$ -gal A/mg protein). That the correction is attributable to  $\alpha$ -gal A taken up via the M6P receptor is demonstrated by its inhibition with mannose-6-phosphate (BRS-11 insert + M6P).

Table 7. Correction of Fabry Fibroblasts by Human Fibroblasts Expressing  $\alpha$ -Gal A

$\alpha$ -Gal A Activity (units/mg total protein)

Time	no insert	HF insert	BRS-11 insert	BRS-11 insert+M6P
Day 1	2 $\pm$ 1	2 $\pm$ 1	13 $\pm$ 1	4 $\pm$ 1
Day 2	2 $\pm$ 1	2 $\pm$ 1	40 $\pm$ 11	6 $\pm$ 2
Day 3	2 $\pm$ 1	5 $\pm$ 1	85 $\pm$ 1	9 $\pm$ 1

#### H. Utility of Other Cell Types

Other cell types can be used in the method described herein. The cells can be obtained from a variety of tissues and include all cell types that can be

- 42 -

maintained in culture. For example, primary and secondary cells that can be transfected by the present method include human fibroblasts, keratinocytes, epithelial cells (e.g., mammary or intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes and bone marrow cells), muscle cells, and precursors of these somatic cell types. Fibroblasts are of particular interest. Primary cells are preferably obtained from the individual to whom the transfected primary or secondary cells are to be administered, so that they will not be rejected by the patient's immune system. However, if proper attention is paid to avoiding or suppressing immunorejection (as described below), cells of a human donor other than the patient can be used as well. This would permit use of cells from an standardized, established, stably transfected cell line in all patients.

#### I. Administration of $\alpha$ -Gal A-Expressing Cells

The cells described above may be introduced into an individual, through various standardized routes of administration, so that they will reside in, for example, the renal subcapsule, a subcutaneous compartment, the central nervous system, the intrathecal space, the liver, the intraperitoneal cavity, or within a muscle. The cells may also be injected intravenously or intra-arterially so that they circulate within the individual's bloodstream. Once implanted in the individual, the transfected cells will produce and secrete the therapeutic product, glycosylated human  $\alpha$ -gal A.

The number of genetically modified cells that will be introduced into the individual will vary, but can be determined by skilled artisans. The age, weight, sex, and general physical condition of each patient, as well



- 43 -

as the volume of distribution, the half-life and bioavailability of the enzyme, and the *in vivo* productivity of the genetically modified cells, will be among the primary considerations in determining dosage  
5 and route of administration. Typically, between one million and one billion cells will be used, with expression levels ranging from 100-100,000 units per 10<sup>6</sup> cells per day. If necessary, the procedure may be repeated or modified until the desired result, for  
10 example, relief from the symptoms associated with Fabry disease, is achieved.

As described above, the cells used will generally be patient-specific, i.e., obtained from the individual to whom the transfected primary or secondary  
15 cells are to be administered, so that they will not be rejected by the patient's immune system. If, however, this scenario is not possible or desirable, cells may be obtained from another individual, genetically modified as described herein, and implanted into the patient who is  
20 suffering from  $\alpha$ -gal A deficiency.

The use of cells from an individual other than the recipient might require administration of an immunosuppressant, alteration of histocompatibility antigens, or use of a barrier device to prevent rejection  
25 of the implanted cells. The barrier device will be made of a material (e.g., a membrane such as XM-50 from Amicon, Beverly, MA) that permits the secreted product to pass into the recipient's circulation or tissues, but prevents contact between the implanted cells and the  
30 recipient's immune system, and thus prevents an immune response to (and possible rejection of) the cells by the recipient. For further guidance regarding gene therapy, see Selden et al. (WO 93/09222).

The cells may alternatively be embedded in a  
35 matrix or gel material, such as described in co-owned

- 44 -

U.S.S.N. 08/548/002, which describes the use of hybrid matrix implants, or in Jain et al. (PCT application WO 95/19430), which describes macroencapsulation of secretory cells in a hydrophilic gel material (each of which is hereby incorporated by reference).

J. Pharmaceutical Formulation for Conventional Administration of  $\alpha$ -Gal A Protein

The  $\alpha$ -gal A protein that is expressed and secreted by stably transfected (or otherwise genetically modified) human cells and purified as described herein may be administered to individuals who produce insufficient or defective  $\alpha$ -gal A protein. The protein may be administered in a pharmaceutically acceptable carrier, at a pH below 6.5, e.g. in a formulation as described in Example III. Examples of excipients which may be included with the formulation are buffers such as citrate buffer, phosphate buffer, acetate buffer, and bicarbonate buffer, amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins, such as serum albumin and gelatin, EDTA, sodium chloride, liposomes, polyvinylpyrrolidone, mannitol, sorbitol, glycerol, propylene glycol and polyethylene glycol (e.g., PEG-4000, PEG-6000). The route of administration may be, for example, intravenous, intra-arterial, subcutaneous, intraperitoneal, intracerebral, intramuscular, intrapulmonary, or transmucosal. The route of administration and the amount of protein delivered will be determined by factors that are well within the ability of skilled artisans to assess. Furthermore, skilled artisans are aware that the route of administration and dosage of a therapeutic protein may be varied for a given patient until a therapeutic dosage level is obtained. Typically, doses of  $\alpha$ -gal A of 0.01-100 mg/kg of body weight will be administered. It is expected that

- 45 -

regularly repeated doses of the protein will be necessary over the life of the patient.

K. Treatment of Other Conditions  
Caused by Enzyme Deficiencies

5 It is likely that other conditions caused by deficiencies in lysosomal storage enzymes other than  $\alpha$ -gal A will be amenable to treatment by methods comparable to those described herein. In these cases, DNA which encodes a functional form of the deficient  
 10 enzyme would be substituted for the DNA encoding  $\alpha$ -gal A in the expression constructs disclosed herein. Examples of enzyme deficiency syndromes that have been identified, and that may be amenable to treatment as described herein, are shown in Table 8. The information in this  
 15 Table is taken from E. Neufeld (Ann. Rev. Biochem. 60:257-280, 1991), which is hereby incorporated by reference.

Table 8. Summary of Lysosomal Storage Disorders

---

20 Disorder	Primary deficiency [secondary deficiency]
<u>Disorders of sphingolipid degradation</u>	
Fabry disease	$\alpha$ -galactosidase
Farber disease	ceramidase
25 Gaucher disease	glucocerebrosidase
G <sub>M1</sub> gangliosidosis	$\beta$ -galactosidase

- 46 -

G <sub>M2</sub> gangliosidoses subunit	$\beta$ -hexosaminidase, $\alpha$ -
Tay-Sachs disease	[hexosaminidase A]
5 Sandhoff disease subunit	$\beta$ -hexosaminidase, $\beta$ - [hexosaminidases A and B]
Activator deficiency Krabbe disease	G <sub>M2</sub> activator galactosylceramidase
10 Metachromatic leukodystrophy enzyme-deficient form activator-deficient form activator/saposin	arylsulfatase A sulfatide
Mucopolipidosis IV	primary defect unknown [ganglioside sialidase]
15 Multiple sulfatase deficiency sulfatases]	primary defect unknown [deficiency of all
Niemann-Pick disease	sphingomyelinase
20 Schindler disease acetylgalactosaminidase	$\alpha$ -N-
<u>Disorders of glycoprotein degradation</u>	
Aspartylglycosaminuria	aspartylglycosaminidase
Fucosidosis	$\alpha$ -L-fucosidase

- 47 -

	Galactosialidosis protein/cathepsin sialidase]	protective  [ $\beta$ -galactosidase and
5	$\alpha$ -Mannosidosis	$\alpha$ -mannosidase
	$\beta$ -Mannosidosis	$\beta$ -mannosidase
	Sialidosis	sialidase
	<u>Disorders of glycosaminoglycan degradation</u>	
	Hunter syndrome	iduronate sulfatase
10	Hurler and Scheie syndromes	$\alpha$ -L-iduronidase
	Maroteaux-Lamy syndrome sulfatase/arylsulfatase	GalNAc 4-
	Morquio syndrome	
	A-subtype	Gal 6-sulfatase
15	B-subtype	$\beta$ -galactosidase
	Sanfilippo syndrome	
	A-subtype	heparan N-sulfatase
	B-subtype	$\alpha$ -N-acetylglucosaminidase
	C-subtype	AcetylCoA: glucosamine
20		N-acetyltransferase

- 48 -

D-subtype	GlcNAc 6-sulfatase
Sly syndrome	$\beta$ -glucuronidase
<u>Other single enzyme deficiency disorders</u>	

Pompe disease (glycogenosis II)	$\alpha$ -glucosidase
5 Wolman disease	acid lipase

## Disorders of lysosomal enzyme biosynthesis

I-cell disease and pseudoHurler 6-phospho-N-acetylglucosamine transferase

10 polydystrophy [mislocalization of many lysosomal enzymes]

## Disorders of lysosomal membrane transport

Cystinosis	cystine transport
15 Sialic storage and Salla diseases	sialic acid transport

- 49 -

V. Other Embodiments

The invention described herein has been exemplified in part by methods of treatment that employ cells which express a particular gene product following  
5 transfection, i.e., after introduction of a construct encoding the gene product and having regulatory elements that control expression of the coding sequence. These methods may also be carried out using cells that have been genetically modified by other procedures, including  
10 gene targeting and gene activation (see Treco et al. (WO 95/31560, herein incorporated by reference; see also Selden et al. WO 93/09222).

The hGH signal peptide can be used with heterologous proteins other than  $\alpha$ -gal A, to increase the  
15 level of expression and secretion of the heterologous protein. Examples of such proteins include  $\alpha$ -1 antitrypsin, antithrombin III, apolipoprotein E, apolipoprotein A-1, blood clotting factors V, VII, VIII, IX, X, and XIII, bone growth factor-2, bone growth  
20 factor-7, calcitonin, catalytic antibodies, DNase, erythropoietin, FSH- $\beta$ , globins, glucagon, glucocerebrosidase, G-CSF, GM-CSF, growth hormone, immune response modifiers, immunoglobulins, insulin, insulinotropin, insulin-like growth factors,  
25 interferon- $\beta$ , interferon- $\beta$  nerve growth factors, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-11, interleukin-12, IL-2 receptor, IL-1 receptor antagonists, low density lipoprotein receptor, M-CSF, parathyroid hormone, protein  
30 kinase C, soluble CD4, superoxide dismutase, tissue plasminogen activator, TGF- $\beta$ , tumor necrosis factor, TSH $\beta$ , tyrosine hydroxylase, and urokinase.

Other embodiments are within the following claims.

50

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Transkaryotic Therapies, Inc.
- (ii) TITLE OF INVENTION: THERAPY FOR  $\alpha$ -GALACTOSIDASE A DEFICIENCY
- (iii) NUMBER OF SEQUENCES: 28
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Fish & Richardson, P.C.
  - (B) STREET: 225 Franklin Street
  - (C) CITY: Boston
  - (D) STATE: MA
  - (E) COUNTRY: USA
  - (F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/712,614
  - (B) FILING DATE: 13-SEP-1996
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Fraser, Janis K.
  - (B) REGISTRATION NUMBER: 34,819
  - (C) REFERENCE/DOCKET NUMBER: 07236/003WO1
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 617/542-5070
  - (B) TELEFAX: 617/542-8906
  - (C) TELEX: 200154

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGGGCTGTA GCTATGATAA AC

22

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:



51

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCTAGCTGAA GCAAAACAGT G

21

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATTGGTCCGC CCCTGAGGT

19

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGATGCAGGA ATCTGGCTCT

20

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTTGGATCC ACCATGGCTA

20

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTTTGCCGGC ACTGCCCTCT TGAA

24

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid

52

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTTTCAGCTG GACAATGGAT TGGC

24

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTTTGCTAGC TGGCGAATCC

20

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTTTGGATCC GTGTCCCATATA GTGTTTCCAA

30

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTTGGATCC GCAGTCGTGG CCACTACC

28

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTAGTCCTAG GA

12

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:  
TTTTGAGCAC AGAGCCTCGC CT 22
- (2) INFORMATION FOR SEQ ID NO:13:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:  
TTTTGGATCC GGTGAGCTGC GAGAATAGCC 30
- (2) INFORMATION FOR SEQ ID NO:14:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 76 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:  
GGGCCCCCAG CCCAGCCCT CCCATTGGTG GAGGCCCTTT TGGAGGCACC CTAGGGCCAG 60  
GAAACTTTTG CCGTAT 76
- (2) INFORMATION FOR SEQ ID NO:15:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 69 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:  
AAATAGGGCA GATCCGGGCT TTATTATTTT AGCACCACGG CCGCGAGAC CGCGTCCGCC 60  
CCGCGAGCA 69
- (2) INFORMATION FOR SEQ ID NO:16:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:  
TGCCCTATTT ATACGGCAAA AGTTTCCTGG CCCTAGGGTG CCTCCAAAAG GGCCTCCACC 60  
AATGGGAGGG CTGGGGCTGG GGGCCC 86
- (2) INFORMATION FOR SEQ ID NO:17:
- (i) SEQUENCE CHARACTERISTICS:

54

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGCGGGGCGG ACGCGGTCTC GGCGGCCGTG GTGCTAAAAT AATAAGCCC GGATC 55

(2) INFORMATION FOR SEQ ID NO:18:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1343 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCGCGGGGAAA TTTATGCTGT CCGGTCACCG TGACAATGCA GCTGAGGAAC CCAGAACTAC 60  
 ATCTGGGCTG CGCGCTTGCG CTTGCTTCC TGGCCCTCGT TTCCTGGGAC ATCCCTGGGG 120  
 CTAGAGCACT GGACAATGGA TTGGCAAGGA CGCCTACCAT GGGCTGGCTG CACTGGGAGC 180  
 GCTTCATGTG CAACCTTGAC TGCCAGGAAG AGCCAGATTC CTGCATCAGT GAGAAGCTCT 240  
 TCATGGAGAT GGCAGAGCTC ATGGTCTCAG AAGGCTGGAA GGATGCAGGT TATGAGTACC 300  
 TCTGCATTGA TGA CTGTTGG ATGGCTCCCC AAAGAGATTC AGAAGGCAGA CTTCAGGCAG 360  
 ACCCTCAGCG CTTTCCTCAT GGGATTGCGC AGCTAGCTAA TTATGTTTAC AGCAAAGGAC 420  
 TGAAGCTAGG GATTTATGCA GATGTTGGAA ATAAACCTG CGCAGGCTTC CCTGGGAGTT 480  
 TTGGATACTA CGACATTGAT GCCCAGACCT TTGCTGACTG GGGAGTAGAT CTGCTAAAAT 540  
 TTGATGGTTG TTA CTGTGAC AGTTTGGAAT ATTTGCCAGA TGGTTATAAG CACATGTCCT 600  
 TGGCCCTGAA TAGGACTGGC AGAAGCATTG TGTACTCCTG TGAGTGGCCT CTTTATATGT 660  
 GGCCCTTTCA AAAGCCCAAT TATACAGAAA TCCGACAGTA CTGCAATCAC TGGCGAAATT 720  
 TTGCTGACAT TGATGATTCC TGGAAAAGTA TAAAGAGTAT CTTGGACTGG ACATCTTTTA 780  
 ACCAGGAGAG AATTGTTGAT GTTCTGGAC CAGGGGGTTG GAATGACCCA GATATGTTAG 840  
 TGATTGGCAA CTTTGGCCTC AGCTGGAATC AGCAAGTAAC TCAGATGGCC CTCTGGGCTA 900  
 TCATGGCTGC TCCTTTATTG ATGTCTAATG ACCTCCGACA CATCAGCCCT CAAGCCAAAG 960  
 CTCTCCTTCA GGATAAGGAC GTAATTGCCA TCAATCAGGA CCCCTTGGGC AAGCAAGGGT 1020  
 ACCAGCTTAG ACAGGGAGAC AACTTTGAAG TGTGGGAACG ACCTCTCTCA GGCTTAGCCT 1080  
 GGGCTGTAGC TATGATAAAC CGGCAGGAGA TTGGTGGACC TCGCTCTTAT ACCATCGCAG 1140  
 TTGCTTCCCT GGGTAAAGGA GTGGCCTGTA ATCCTGCCTG CTTTCATACA CAGCTCCTCC 1200  
 CTGTGAAAAG GAAGCTAGGG TTCTATGAAT GGA CTTC AAG GTTAAGAAGT CACATAAATC 1260  
 CCACAGGCAC TGTTTGTCTT CAGCTAGAAA ATACAATGCA GATGTCATTA AAAGACTTAC 1320

TTTAAAAAAA AAAAAAACTC GAG

1343

## (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 210 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTGGGCTGTA GCTATGATAA ACCGGCAGGA GATTGGTGGA CCTCGCTCTT ATACCATCGC	60
AGTTGCTTCC CTGGGTAAAG GAGTGGCCTG TAATCCTGCC TGCTTCATCA CACAGCTCCT	120
CCCTGTGAAA AGGAAGCTAG GGTCTATGA ATGGAATTCA AGGTTAAGAA GTCACATAAA	180
TCCACAGGC ACTGTTTTGC TTCAGCTAGA	210

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 268 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATTGGTCCGC CCCTGAGGTT AATCTTAAAA GCCCAGGTTA CCCGCCGAAA TTTATGCTGT	60
CCGGTCACCG TGACAATGCA GCTGAGGAAC CCAGAACTAC ATCTGGGCTG CGCGCTTGCG	120
CTTCGCTTCC TGGCCCTCGT TTCCTGGGAC ATCCCTGGGG CTAGAGCACT GGACAATGGA	180
TTGGCAAGGA CGCCTACCAT GGGCTGGCTG CACTGGGAGC GCTTCATGTG CAACCTTGAC	240
TGCCAGGAAG AGCCAGATTC CTGCATCA	268

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 26 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met	Ala	Thr	Gly	Ser	Arg	Thr	Ser	Leu	Leu	Leu	Ala	Phe	Gly	Leu	Leu
1				5				10						15	
Cys	Leu	Pro	Trp	Leu	Gln	Glu	Gly	Ser	Ala						
		20						25							

## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 78 base pairs

56

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGGCTACAG GCTCCCGGAC GTCCCTGCTC CTGGCTTTTG GCCTGCTCTG CCTGCCCTGG 60  
 CTTCAAGAGG GCAGTGCC 78

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTTTGGATCC CTCGAGGACA TTGATTATTG ACTAG 35

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TTTTGGATCC CGTGTCAAGG ACGGTGAC 28

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1197 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTGGACAATG GATTGGCAAG GACGCCTACC ATGGGCTGGC TGCACTGGGA GCGCTTCATG 60  
 TGCAACCTTG ACTGCCAGGA AGAGCCAGAT TCCTGCATCA GTGAGAAGCT CTTATGGAG 120  
 ATGGCAGAGC TCATGGTCTC AGAAGGCTGG AAGGATGCAG GTTATGAGTA CCTCTGCATT 180  
 GATGACTGTT GGATGGCTCC CCAAGAGAT TCAGAAGGCA GACTTCAGGC AGACCCTCAG 240  
 CGCTTTCCTC ATGGGATTGG CCAGCTAGCT AATTATGTTT ACAGCAAAGG ACTGAAGCTA 300  
 GGGATTTATG CAGATGTTGG AAATAAAACC TGCGCAGGCT TCCCTGGGAG TTTTGGATAC 360  
 TACGACATTG ATGCCAGAC CTTTGCTGAC TGGGGAGTAG ATCTGCTAAA ATTTGATGGT 420  
 TGTTACTGTG ACAGTTTGGG AAATTTGGCA GATGTTATA AGCACATGTC CTTGGCCCTG 480

57

```

AATAGGACTG GCAGAAGCAT TGTGTACTCC TGTGAGTGGC CTCTTTATAT GTGGCCCTTT      540
CAAAGCCCA ATTATACAGA AATCCGACAG TACTGCAATC ACTGGCGAAA TTTTGCTGAC      600
ATTGATGATT CCTGGAAGAG TATAAAGAGT ATCTTGGACT GGACATCTTT TAACCAGGAG      660
AGAATTGTTG ATGTTGCTGG ACCAGGGGGT TGAATGACC CAGATATGTT AGTGATTGGC      720
AACTTTGGCC TCAGCTGGAA TCAGCAAGTA ACTCAGATGG CCCTCTGGGC TATCATGGCT      780
GCTCCTTTAT TCATGTCTAA TGACCTCCGA CACATCAGCC CTCAAGCCAA AGCTCTCCTT      840
CAGGATAAGG ACGTAATTGC CATCAATCAG GACCCCTTGG GCAAGCAAGG GTACCAGCTT      900
AGACAGGGAG ACAACTTTGA AGTGTGGGAA CGACCTCTCT CAGGCTTAGC CTGGGCTGTA      960
GCTATGATAA ACCGGCAGGA GATTGGTGGG CCTCGCTCTT ATACCATCGC AGTTGCTTCC     1020
CTGGGTAAAG GAGTGGCCTG TAATCCTGCC TGCTTCATCA CACAGCTCCT CCCTGTGAAA     1080
AGGAAGCTAG GGTTCATGA ATGGACTTCA AGGTTAAGAA GTCACATAAA TCCCACAGGC     1140
ACTGTTTTGC TTCAGCTAGA AAATACAATG CAGATGTCAT TAAAAGACTT ACTTTAA       1197

```

## (2) INFORMATION FOR SEQ ID NO:26:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 398 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```

Leu Asp Asn Gly Leu Ala Arg Thr Pro Thr Met Gly Trp Leu His Trp
 1           5           10
Glu Arg Phe Met Cys Asn Leu Asp Cys Gln Glu Glu Pro Asp Ser Cys
          20           25           30
Ile Ser Glu Lys Leu Phe Met Glu Met Ala Glu Leu Met Val Ser Glu
          35           40           45
Gly Trp Lys Asp Ala Gly Tyr Glu Tyr Leu Cys Ile Asp Asp Cys Trp
          50           55           60
Met Ala Pro Gln Arg Asp Ser Glu Gly Arg Leu Gln Ala Asp Pro Gln
          65           70           75           80
Arg Phe Pro His Gly Ile Arg Gln Leu Ala Asn Tyr Val His Ser Lys
          85           90           95
Gly Leu Lys Leu Gly Ile Tyr Ala Asp Val Gly Asn Lys Thr Cys Ala
          100          105          110
Gly Phe Pro Gly Ser Phe Gly Tyr Tyr Asp Ile Asp Ala Gln Thr Phe
          115          120          125
Ala Asp Trp Gly Val Asp Leu Leu Lys Phe Asp Gly Cys Tyr Cys Asp
          130          135          140

```

58

Ser Leu Glu Asn Leu Ala Asp Gly Tyr Lys His Met Ser Leu Ala Leu  
 145 150 155 160  
 Asn Arg Thr Gly Arg Ser Ile Val Tyr Ser Cys Glu Trp Pro Leu Tyr  
 165 170 175  
 Met Trp Pro Phe Gln Lys Pro Asn Tyr Thr Glu Ile Arg Gln Tyr Cys  
 180 185 190  
 Asn His Trp Arg Asn Phe Ala Asp Ile Asp Asp Ser Trp Lys Ser Ile  
 195 200 205  
 Lys Ser Ile Leu Asp Trp Thr Ser Phe Asn Gln Glu Arg Ile Val Asp  
 210 215 220  
 Val Ala Gly Pro Gly Gly Trp Asn Asp Pro Asp Met Leu Val Ile Gly  
 225 230 235 240  
 Asn Phe Gly Leu Ser Trp Asn Gln Gln Val Thr Gln Met Ala Leu Trp  
 245 250 255  
 Ala Ile Met Ala Ala Pro Leu Phe Met Ser Asn Asp Leu Arg His Ile  
 260 265 270  
 Ser Pro Gln Ala Lys Ala Leu Leu Gln Asp Lys Asp Val Ile Ala Ile  
 275 280 285  
 Asn Gln Asp Pro Leu Gly Lys Gln Gly Tyr Gln Leu Arg Gln Gly Asp  
 290 295 300  
 Asn Phe Glu Val Trp Glu Arg Pro Leu Ser Gly Leu Ala Trp Ala Val  
 305 310 315 320  
 Ala Met Ile Asn Arg Gln Glu Ile Gly Gly Pro Arg Ser Tyr Thr Ile  
 325 330 335  
 Ala Val Ala Ser Leu Gly Lys Gly Val Ala Cys Asn Pro Ala Cys Phe  
 340 345 350  
 Ile Thr Gln Leu Leu Pro Val Lys Arg Lys Leu Gly Phe Tyr Glu Trp  
 355 360 365  
 Thr Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr Val Leu Leu  
 370 375 380  
 Gln Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp Leu Leu  
 385 390 395

## (2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 338 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATGGCTACAG GTAAGCGCCC CTAAAATCCC TTTGGGCACA ATGTGTCCTG AGGGGAGAGG	60
CAGCGACCTG TAGATGGGAC GGGGGCACTA ACCCTCAGGT TTGGGGCTTC TGAATGTGAG	120



59

TATCGCCATG TAAGCCCAGT ATTTGGCCAA TCTCAGAAAG CTCCTGGTCC CTGGAGGGAT	180
GGAGAGAGAA AAACAAACAG CTCCTGGAGC AGGGAGAGTG CTGGCCTCTT GCTCTCCGGC	240
TCCCTCTGTT GCCCTCTGGT TTCTCCCCAG GCTCCCGGAC GTCCCTGCTC CTGGCTTTTG	300
GCCTGCTCTG CCTGCCCTGG CTTCAAGAGG GCAGTGCC	338

## (2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Leu	Asp	Asn	Gly	Leu	Ala	Arg	Thr	Pro	Thr
1				5					10

- 60 -

What is claimed is:

1. A method of treatment comprising identifying a patient suspected of having an  $\alpha$ -galactosidase A deficiency, and introducing into the patient a human cell  
5 genetically modified to overexpress and secrete human  $\alpha$ -gal A.

2. The method of claim 1, wherein the cell has been transfected in vitro with a DNA molecule having a coding sequence encoding a polypeptide comprising human  
10  $\alpha$ -gal A (SEQ ID NO:26).

3. The method of claim 2, wherein the polypeptide further comprises an heterologous signal peptide.

4. The method of claim 3, wherein the heterologous signal peptide is the human growth hormone  
15 (hGH) signal peptide (SEQ ID NO:21).

5. The method of claim 3, wherein the DNA molecule comprises an intron within the sequence encoding the signal peptide.

6. The method of claim 3, wherein the DNA  
20 molecule comprises an untranslated sequence of at least 6 nucleotides lying immediately 3' to the termination codon of the coding sequence, said untranslated sequence comprising a polyadenylation site.

7. A DNA molecule comprising  
25 a first sequence encoding the hGH signal peptide (SEQ ID NO:21), said first sequence comprising an intron; and,

- 61 -

linked to the 3' end of the first sequence, a second sequence encoding a polypeptide comprising human  $\alpha$ -gal A.

8. The DNA molecule of claim 7, further  
5 comprising a 3' untranslated sequence comprising a polyadenylation site.

9. An expression construct comprising the DNA molecule of claim 7 and suitable for expression in a human cell.

10 10. A cultured human cell containing a DNA molecule that (a) encodes a polypeptide comprising human  $\alpha$ -gal A linked to an heterologous signal peptide, and (b) permits expression of the polypeptide in the cell.

11. The cell of claim 10, said cell being a  
15 fibroblast.

12. The cell of claim 10, wherein said cell is selected from the group consisting of an epithelial cell, an endothelial cell, a bone marrow cell, a glial cell, a hepatocyte, a keratinocyte, a myocyte, a neuron, or a  
20 precursor of these cell types.

13. The cell of claim 10, wherein said signal peptide is the hGH signal peptide (SEQ ID NO:21).

14. A clonal cell strain consisting essentially of a plurality of cultured human cells transfected with a  
25 DNA molecule that (a) encodes a polypeptide comprising human  $\alpha$ -gal A linked to an heterologous signal peptide, and (b) permits overexpression of the polypeptide in the cells.

- 62 -

15. The clonal cell strain of claim 14, said cells being fibroblasts.

16. A clonal cell line consisting essentially of a plurality of immortalized human cells transfected with  
5 a DNA molecule that (a) encodes a polypeptide comprising human  $\alpha$ -gal A linked to an heterologous signal peptide, and (b) permits overexpression of the polypeptide in the cells.

17. The clonal cell line of claim 16, wherein  
10 said cell is selected from the group consisting of a Bowes melanoma cell, a Daudi cell, a HeLa cell, an HL-60 cell, an HT1080 cell, a Jurkat cell, a KB carcinoma cell, a K-562 leukemia cell, an MCF-7 breast cancer cell, a MOLT-4 cell, a Namalwa cell, a Raji cell, an RPMI 8226  
15 cell, a U-937 cell, a WI-38VA13 (subline 2R4) cell, and a 2780AD ovarian carcinoma cell.

18. A protein comprising (a) the hGH signal peptide (SEQ ID NO:21) linked by a peptide bond to (b) human  $\alpha$ -gal A.

20 19. A process for making glycosylated human  $\alpha$ -gal A, comprising  
culturing the cell of claim 11 under conditions permitting expression of human  $\alpha$ -gal A from said DNA molecule and secretion of glycosylated human  $\alpha$ -gal A into  
25 the culture medium of the cell; and  
isolating glycosylated human  $\alpha$ -gal A from the culture medium.

20. Purified glycosylated human  $\alpha$ -gal A made by the process of claim 19.

- 63 -

21. A process for purifying human  $\alpha$ -gal A from a sample, comprising a first chromatography step wherein the sample is passed over a hydrophobic interaction resin.

5           22. The process of claim 21, wherein the functional moiety on the resin comprises a butyl group.

          23. A process for making glycosylated human  $\alpha$ -gal A, comprising  
          culturing the cell of claim 16 under conditions  
10   permitting expression of human  $\alpha$ -gal A from said DNA molecule and secretion of glycosylated human  $\alpha$ -gal A into the culture medium of the cell; and  
          isolating glycosylated human  $\alpha$ -gal A from the culture medium.

15           24. The process of claim 21, further comprising the step of passing the sample over a second resin selected from the group consisting of an immobilized heparin resin, hydroxyapatite, an anion exchange resin, and a size exclusion resin.

20           25. A method of treatment comprising identifying a patient suspected of having an  $\alpha$ -gal A deficiency, and  
          administering to the patient the purified glycosylated human  $\alpha$ -gal A of claim 20.

25           26. A therapeutic composition comprising pharmaceutically acceptable purified human  $\alpha$ -gal A having N-linked mannose-6-phosphate in a pharmaceutically acceptable carrier.

- 64 -

27. A therapeutic composition comprising the purified human  $\alpha$ -gal A of claim 20 and a pharmaceutically acceptable excipient.

28. The therapeutic composition of claim 27,  
5 formulated at pH 6.5 or lower.

29. The therapeutic composition of claim 28, wherein the pharmaceutically acceptable excipient is human serum albumin.

30. A DNA molecule encoding a protein comprising  
10 the signal peptide of hGH (SEQ ID NO:21) linked to a polypeptide other than hGH.

31. The DNA molecule of claim 30, wherein the DNA molecule comprises an intron within the sequence encoding the signal peptide.

15 32. A cultured human cell containing a DNA molecule that (a) encodes a polypeptide comprising human  $\alpha$ -gal A, and (b) permits overexpression of the polypeptide in the cell.

33. A process for making glycosylated human  
20  $\alpha$ -gal A, comprising culturing a human cell containing a DNA molecule that encodes a polypeptide comprising human  $\alpha$ -gal A under conditions permitting expression of human  $\alpha$ -gal A from said DNA molecule.

34. The therapeutic composition of claim 26  
25 further comprising a pharmaceutically acceptable excipient.

- 65 -

35. A process for purifying human  $\alpha$ -gal A from a sample comprising passing said sample over a hydrophobic interaction resin comprising a butyl group as a functional moiety.

1/11

CTGGGCTGTAGCTATGATAAACCGGCAGGA  
GATTGGTGGACCTCGCTCTTATACCATCGCA  
GTTGCTTCCCTGGGTAAAGGAGTGGCCTGTA  
ATCCTGCCTGCTTCATCACACAGCTCCTCCCT  
GTGAAAAGGAAGCTAGGGTTCTATGAATGGA  
CTTCAAGGTTAAGAAGTCACATAAATCCCAC  
AGGCACTGTTTTGCTTCAGCTAGA

**FIG. 1**



2/11

ATTGGTCCGCCCCTGAGGTTAATCTTAAAAG

SacII

CCCAGGTTACCCGCGGAAATTTATGCTGTC  
CGGTCACCGTGACAATGCAGCTGAGGAACC  
CAGAACTACATCTGGGCTGCGCGCTTGGCGCT  
TCGCTTCCTGGCCCTCGTTTCCTGGGACATC  
CCTGGGGCTAGAGCACTGGACAATGGATTG

NcoI

GCAAGGACGCCTACCATGGGCTGGCTGCAC  
TGGGAGCGCTTCATGTGCAACCTTGACTGCC  
AGGAAGAGCCAGATTCCTGCATCA

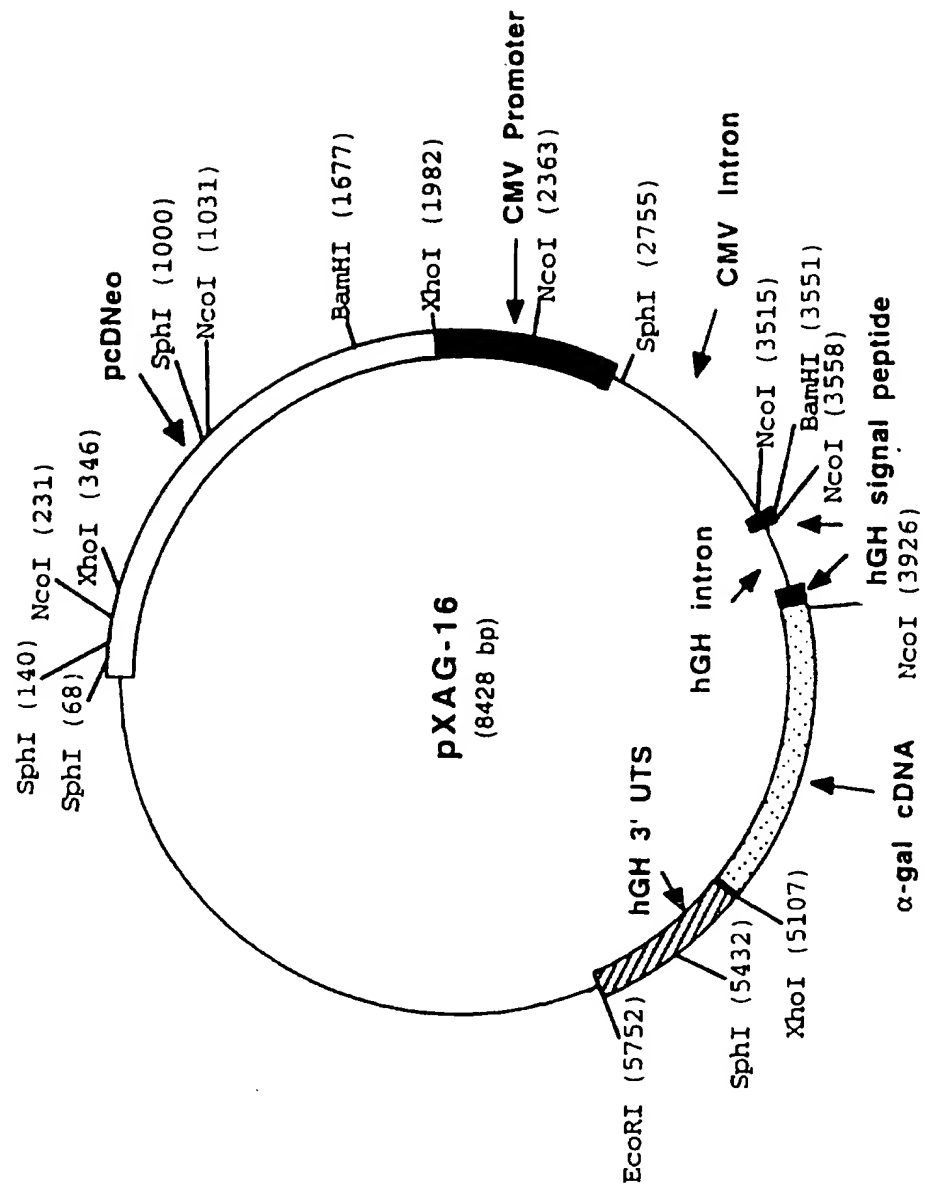
**FIG. 2**

3/11

1 CCGCGGGAAA TTTATGCTGT CCGGTCACCG TGACAATGCA GCTGAGGAAC CCAGAACTAC  
61 ATCTGGGCTG CGCGCTTGCG CTTGCTTCC TGGCCCTCGT TTCCTGGGAC ATCCCTGGGG  
121 CTAGAGCACT GGACAATGGA TTGGCAAGGA CGCCTACCAT GGGCTGGCTG CACTGGGAGC  
181 GCTTCATGTG CAACCTTGAC TGCCAGGAAG AGCCAGATTC CTGCATCAGT GAGAAGCTCT  
241 TCATGGAGAT GGCAGAGCTC ATGGTCTCAG AAGGCTGGAA GGATGCAGGT TATGAGTACC  
301 TCTGCATTGA TGA CTGTTGG ATGGCTCCCC AAAGAGATTC AGAAGGCAGA CTTCAGGCAG  
361 ACCCTCAGCG CTTTCTCAT GGGATTCCGC AGCTAGCTAA TTATGTTTAC AGCAAAGGAC  
421 TGAAGCTAGG GATTTATGCA GATGTTGGAA ATAAAACCTG CGCAGGCTTC CCTGGGAGTT  
481 TTGGATACTA CGACATTGAT GCCCAGACCT TTGCTGACTG GGGAGTAGAT CTGCTAAAAT  
541 TTGATGGTTG TTACTGTGAC AGTTTGGAAA ATTTGGCAGA TGGTTATAAG CACATGTCCT  
601 TGGCCCTGAA TAGGACTGGC AGAAGCATTG TGTACTCCTG TGAGTGGCCT CTTTATATGT  
661 GGCCCTTTCA AAAGCCCAAT TATACAGAAA TCCGACAGTA CTGCAATCAC TGGCGAAATT  
721 TTGCTGACAT TGATGATTCC TGGAAAAGTA TAAAGAGTAT CTTGGACTGG ACATCTTTTA  
781 ACCAGGAGAG AATTGTTGAT GTTGCTGGAC CAGGGGGTTG GAATGACCCA GATATGTTAG  
841 TGATTGGCAA CTTTGGCCTC AGCTGGAATC AGCAAGTAAC TCAGATGGCC CTCTGGGCTA  
901 TCATGGCTGC TCCTTTATTC ATGTCTAATG ACCTCCGACA CATCAGCCCT CAAGCCAAAG  
961 CTCTCCTTCA GGATAAGGAC GTAATTGCCA TCAATCAGGA CCCCTTGGGC AAGCAAGGGT  
1021 ACCAGCTTAG ACAGGGAGAC AACTTTGAAG TGTGGGAACG ACCTCTCTCA GGCTTAGCCT  
1081 GGGCTGTAGC TATGATAAAC CGGCAGGAGA TTGGTGGACC TCGCTCTTAT ACCATCGCAG  
1141 TTGCTTCCCT GGGTAAAGGA GTGGCCTGTA ATCCTGCCTG CTTTCATCACA CAGCTCCTCC  
1201 CTGTGAAAAG GAAGCTAGGG TTCTATGAAT GGACTTCAAG GTTAAGAAGT CACATAAATC  
1261 CCACAGGCAC TGTTTTGCTT CAGCTAGAAA ATACAATGCA GATGTCATTA AAAGACTTAC  
1321 TTTAAAAAAA AAAAAAATC GAG

FIG. 3

4/11



**FIG. 4**

5/11

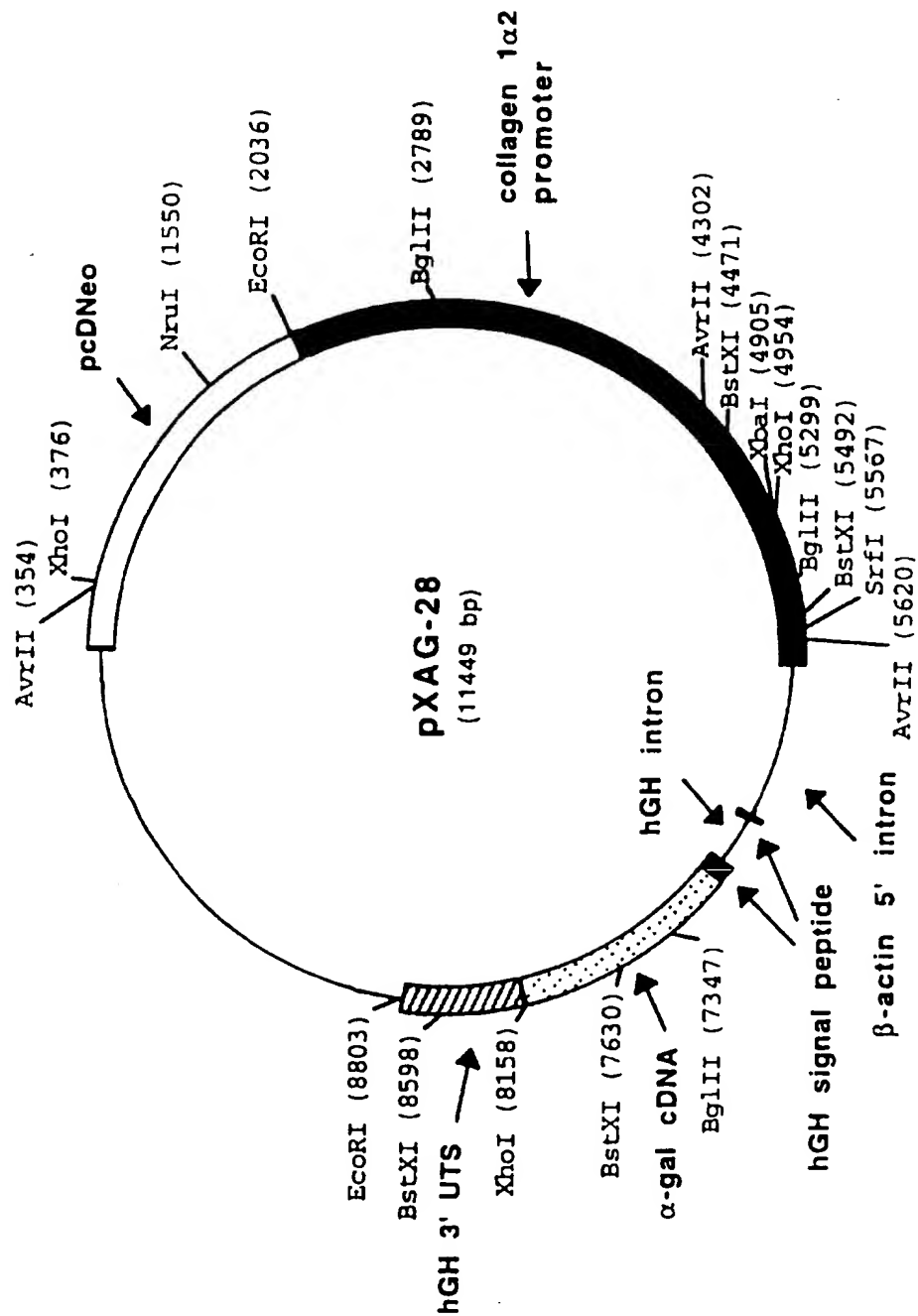


FIG. 5

6/11

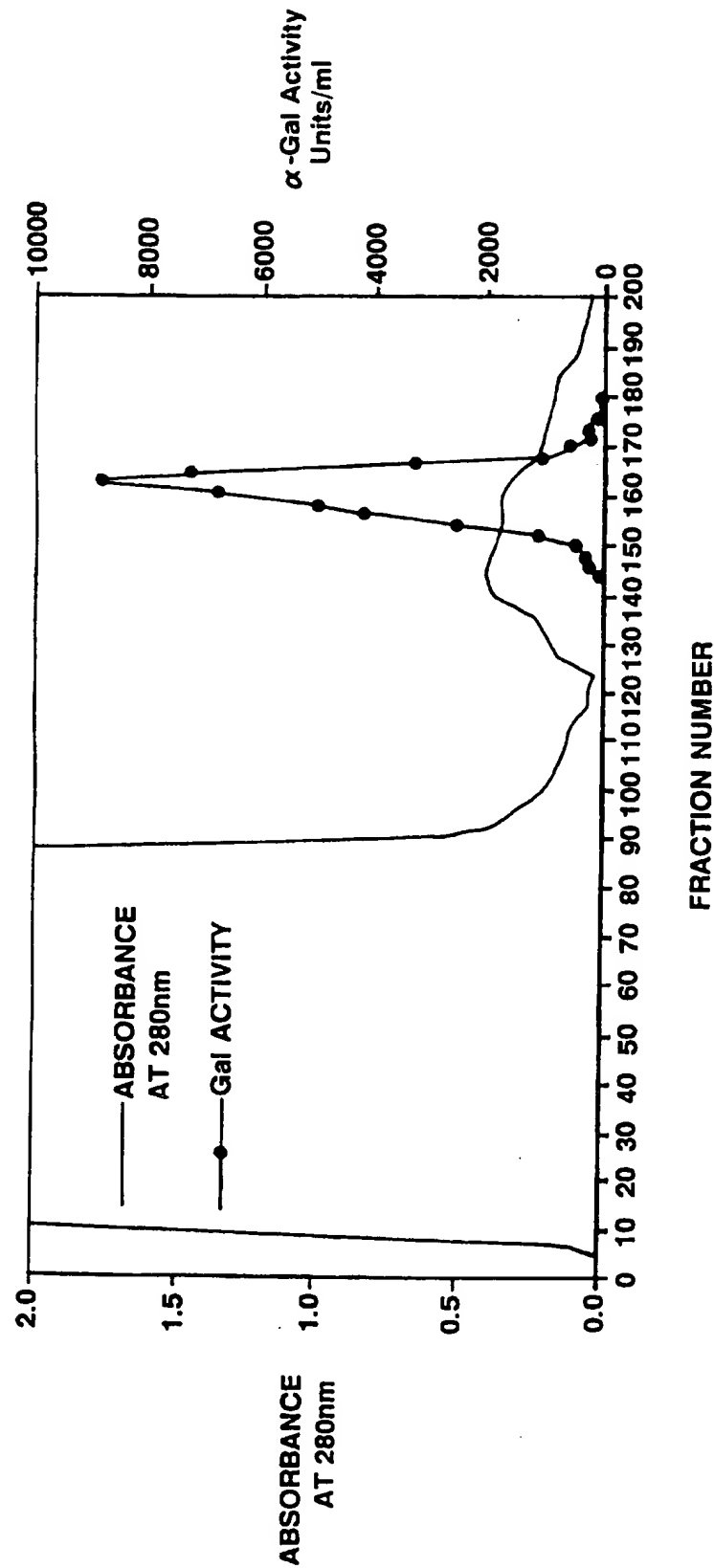


FIG. 6

7/11

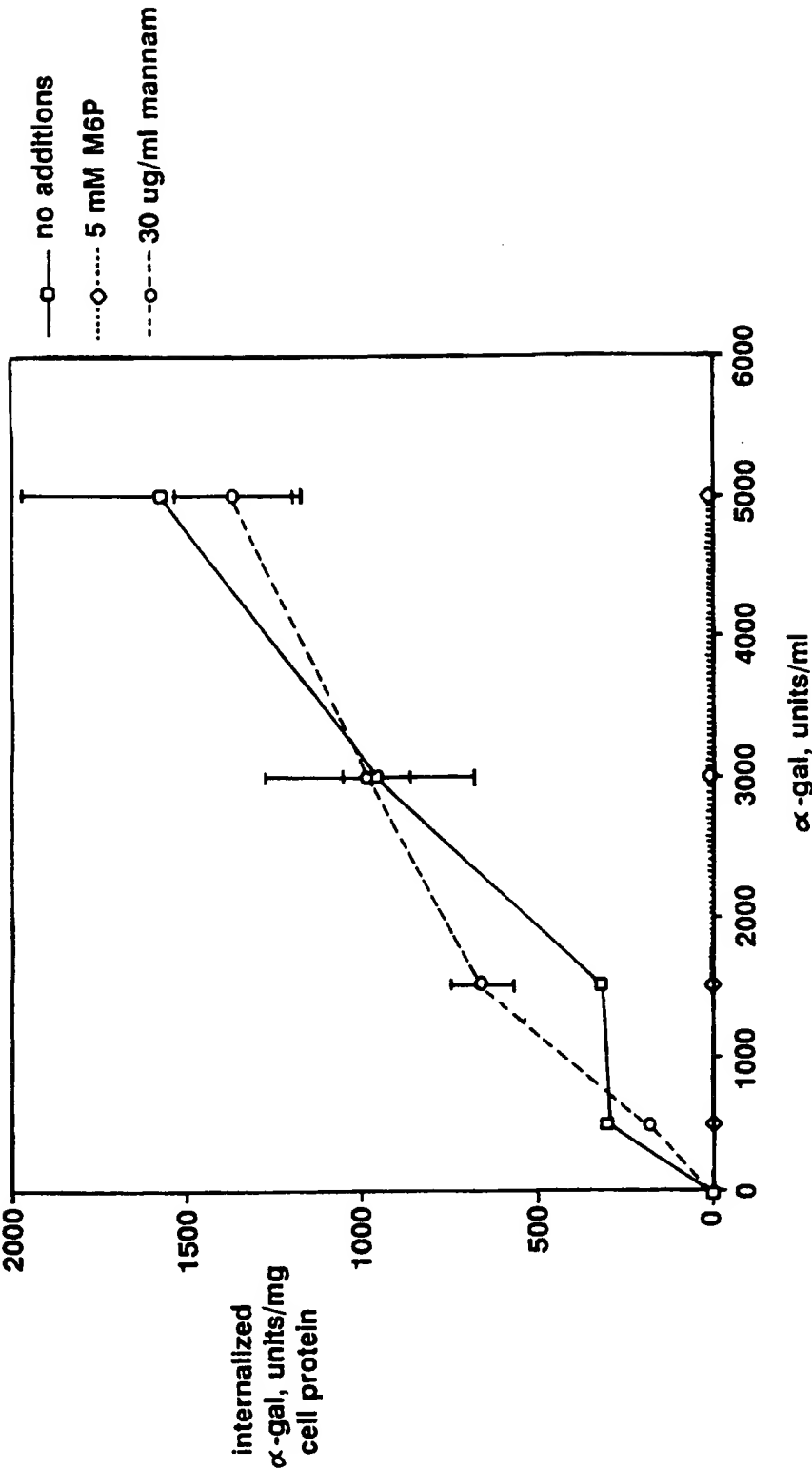


FIG. 7

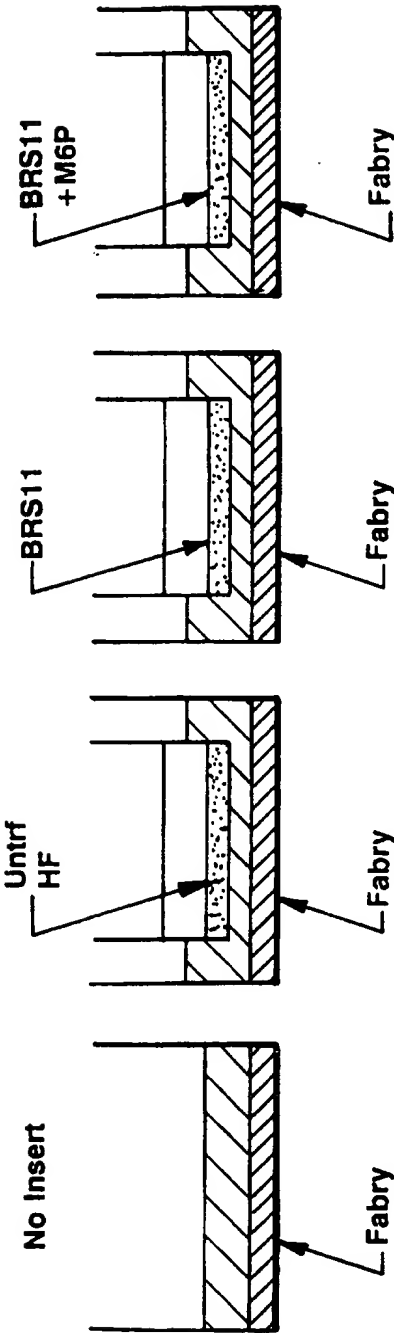


FIG. 8

9/11

Leu	Asp	Asn	Gly	Leu	Ala	Arg	Thr	Pro	Thr	Met	Gly
Trp	Leu	His	Trp	Glu	Arg	Phe	Met	Cys	Asn	Leu	Asp
Cys	Gln	Glu	Glu	Pro	Asp	Ser	Cys	Ile	Ser	Glu	Lys
Leu	Phe	Met	Glu	Met	Ala	Glu	Leu	Met	Val	Ser	Glu
Gly	Trp	Lys	Asp	Ala	Gly	Tyr	Glu	Tyr	Leu	Cys	Ile
Asp	Asp	Cys	Trp	Met	Ala	Pro	Gln	Arg	Asp	Ser	Glu
Gly	Arg	Leu	Gln	Ala	Asp	Pro	Gln	Arg	Phe	Pro	His
Gly	Ile	Arg	Gln	Leu	Ala	Asn	Tyr	Val	His	Ser	Lys
Gly	Leu	Lys	Leu	Gly	Ile	Tyr	Ala	Asp	Val	Gly	Asn
Lys	Thr	Cys	Ala	Gly	Phe	Pro	Gly	Ser	Phe	Gly	Tyr
Tyr	Asp	Ile	Asp	Ala	Gln	Thr	Phe	Ala	Asp	Trp	Gly
Val	Asp	Leu	Leu	Lys	Phe	Asp	Gly	Cys	Tyr	Cys	Asp
Ser	Leu	Glu	Asn	Leu	Ala	Asp	Gly	Tyr	Lys	His	Met
Ser	Leu	Ala	Leu	Asn	Arg	Thr	Gly	Arg	Ser	Ile	Val
Tyr	Ser	Cys	Glu	Trp	Pro	Leu	Tyr	Met	Trp	Pro	Phe
Gln	Lys	Pro	Asn	Tyr	Thr	Glu	Ile	Arg	Gln	Tyr	Cys
Asn	His	Trp	Arg	Asn	Phe	Ala	Asp	Ile	Asp	Asp	Ser
Trp	Lys	Ser	Ile	Lys	Ser	Ile	Leu	Asp	Trp	Thr	Ser
Phe	Asn	Gln	Glu	Arg	Ile	Val	Asp	Val	Ala	Gly	Pro
Gly	Gly	Trp	Asn	Asp	Pro	Asp	Met	Leu	Val	Ile	Gly
Asn	Phe	Gly	Leu	Ser	Trp	Asn	Gln	Gln	Val	Thr	Gln
Met	Ala	Leu	Trp	Ala	Ile	Met	Ala	Ala	Pro	Leu	Phe
Met	Ser	Asn	Asp	Leu	Arg	His	Ile	Ser	Pro	Gln	Ala
Lys	Ala	Leu	Leu	Gln	Asp	Lys	Asp	Val	Ile	Ala	Ile
Asn	Gln	Asp	Pro	Leu	Gly	Lys	Gln	Gly	Tyr	Gln	Leu
Arg	Gln	Gly	Asp	Asn	Phe	Glu	Val	Trp	Glu	Arg	Pro
Leu	Ser	Gly	Leu	Ala	Trp	Ala	Val	Ala	Met	Ile	Asn
Arg	Gln	Glu	Ile	Gly	Gly	Pro	Arg	Ser	Tyr	Thr	Ile
Ala	Val	Ala	Ser	Leu	Gly	Lys	Gly	Val	Ala	Cys	Asn
Pro	Ala	Cys	Phe	Ile	Thr	Gln	Leu	Leu	Pro	Val	Lys
Arg	Lys	Leu	Gly	Phe	Tyr	Glu	Trp	Thr	Ser	Arg	Leu
Arg	Ser	His	Ile	Asn	Pro	Thr	Gly	Thr	Val	Leu	Leu
Gln	Leu	Glu	Asn	Thr	Met	Gln	Met	Ser	Leu	Lys	Asp
Leu	Leu										

FIG. 9



10/11

ATGGCTACAG GTAAGCGCCC CTAAAATCCC TTTGGGCACA  
ATGTGTCCTG AGGGGAGAGG CAGCGACCTG TAGATGGGAC  
GGGGGCACTA ACCCTCAGGT TTGGGGCTTC TGAATGTGAG  
TATCGCCATG TAAGCCCAGT ATTGGGCCAA TCTCAGAAAG  
CTCCTGGTCC CTGGAGGGAT GGAGAGAGAA AAACAAACAG  
CTCCTGGAGC AGGGAGAGTG CTGGCCTCTT GCTCTCCGGC  
TCCCTCTGTT GCCCTCTGGT TTCTCCCCAG GCTCCCGGAC  
 GTCCCTGCTC CTGGCTTTTG GCCTGCTCTG CCTGCCCTGG  
 CTTCAAGAGG GCAGTGCC

**FIG. 10**

ATGGCTACAG GCTCCCGGAC GTCCCTGCTC CTGGCTTTTG  
 GCCTGCTCTG CCTGCCCTGG CTTCAAGAGG GCAGTGCC

**FIG. 11**

Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala  
 Phe Gly Leu Leu Cys Leu Pro Trp Leu Gln Glu Gly  
 Ser Ala

**FIG. 12**

11/11

1 CTGGACAATG GATTGGCAAG GACGCCTACC ATGGGCTGGC TGCACTGGGA GCGCTTCATG  
 61 TGCAACCTTG ACTGCCAGGA AGAGCCAGAT TCCTGCATCA GTGAGAAGCT CTTTCATGGAG  
 121 ATGGCAGAGC TCATGGTCTC AGAAGGCTGG AAGGATGCAG GTTATGAGTA CCTCTGCATT  
 181 GATGACTGTT GGATGGCTCC CCAAAGAGAT TCAGAAAGCA GACTTCAGGC AGACCCCTCAG  
 241 CGCTTTCCTC ATGGGATTCC CCAGCTAGCT AATTATGTTT ACAGCAAAGG ACTGAAGCTA  
 301 GGGATTTATG CAGATGTTGG AAATAAAACC TGCOCAGGCT TCCCTGGGAG TTTTGGATAC  
 361 TACGACATTG ATGCCCAGAC CTTTGCTGAC TGGGGAGTAG ATCTGCTAAA ATTTGATGGT  
 421 TGTTACTGTG ACAGTTTGGG AAATTTGGCA GATGGTTATA AGCACATGTC CTTGGCCCTG  
 481 AATAGGACTG GCAGAAGCAT TGTGTACTCC TGTGAGTGGC CTCTTTATAT GTGGCCCTTT  
 541 CAAAAGCCCA ATTATACAGA AATCCGACAG TACTGCAATC ACTGGCGAAA TTTTGTGAC  
 601 ATTGATGATT CCTGGAAAAG TATAAAGAGT ATCTTGGACT GGACATCTTT TAACCAGGAG  
 661 AGAATTGTTG ATGTTGCTGG ACCAGGGGGT TGGATGACC CAGATATGTT AGTGATTGGC  
 721 AACTTTGGCC TCAGCTGGAA TCAGCAAGTA ACTCAGATGG CCCTCTGGGC TATCATGGCT  
 781 GCTCCTTTAT TCAATGCTAA TGACCTCCGA CACATCAGCC CTCAAGCCAA AGCTCTCCTT  
 841 CAGGATAAGG ACGTAATTGC CATCAATCAG GACCCCTTGG GCAAGCAAGG GTACCAGCTT  
 901 AGACAGGGAG ACAACTTTGA AGTGTGGGAA CGACCTCTCT CAGGCTTAGC CTGGGCTGTA  
 961 GCTATGATAA ACCGGCAGGA GATTGGTGGG CCTCGCTCTT ATACCATCCG AGTGTCTTCC  
 1021 CTGGGTAAAG GAGTGGCCTG TAATCCTGCC TGCTTCATCA CACAGCTCCT CCCTGTGAAA  
 1081 AGGAAGCTAG GGTTCATGTA ATGGACTTCA AGGTTAAGAA GTCACATAAA TCCCACAGGC  
 1141 ACTGTTTTGC TTCAGCTAGA AAATAACAATG CAGATGTGAT TAAAAGACTT ACTTTAA

FIG. 13



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>6</sup> : <b>C12N 9/40, 15/62, 15/56, A61K 38/47, 48/00, C12N 5/10</b></p>	<p><b>A3</b></p>	<p>(11) International Publication Number: <b>WO 98/11206</b></p> <p>(43) International Publication Date: 19 March 1998 (19.03.98)</p>
<p>(21) International Application Number: PCT/US97/16603</p> <p>(22) International Filing Date: 12 September 1997 (12.09.97)</p> <p>(30) Priority Data: 08/712,614 13 September 1996 (13.09.96) US</p> <p>(71) Applicant (for all designated States except US): TRAN- SKARYOTIC THERAPIES, INC. [US/US]; 195 Albany Street, Cambridge, MA 02139 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): SELDEN, Richard, F. [US/US]; 106 Bristol Road, Wellesley, MA 02181 (US). BOROWSKI, Marianne [US/US]; 47 Bartlett Parkway, Winthrop, MA 02152 (US). GILLESPIE, Frances, P. [US/US]; 47 Gleason Road, Lexington, MA 02173 (US). KINOSHITA, Carol, M. [US/US]; 400 Davis Road, Bed- ford, MA 01730 (US). TRECO, Douglas, A. [US/US]; 87 Brantwood Road, Arlington, MA 02174 (US). WILLIAMS, Melanie, D. [US/US]; 7 Malden Street, Natick, MA 01760 (US).</p> <p>(74) Agent: FRASER, Janis, K.; Fish &amp; Richardson PC, 225 Franklin Street, Boston, MA 02110-2804 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW); Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM); European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE); OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report.</i></p> <p>(88) Date of publication of the international search report: 13 August 1998 (13.08.98)</p>
<p>(54) Title: THERAPY FOR <math>\alpha</math>-GALACTOSIDASE A DEFICIENCY</p> <p>(57) Abstract</p> <p>A therapeutic method whereby an individual suspected of having an <math>\alpha</math>-galactosidase A deficiency, such as Fabry disease, is treated either with (1) human cells that have been genetically modified to overexpress and secrete human <math>\alpha</math>-gal A, or (2) purified human <math>\alpha</math>-gal A obtained from cultured, genetically modified human cells.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/US 97/16603

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N9/40 C12N15/62 C12N15/56 A61K38/47 A61K48/00  
C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MEDIN JA ET AL: "Correction in trans for Fabry disease: expression, secretion and uptake of alpha-galactosidase A in patient-derived cells driven by a high-titer recombinant retroviral vector." PROC NATL ACAD SCI U S A, JUL 23 1996, 93 (15) P7917-22, UNITED STATES, XP002052041	10-12, 14-16, 19,20, 23,25
Y	see the whole document	7-9,13, 17,18,26
Y	--- EP 0 307 285 A (SANOFI SA) 15 March 1989 see examples 1,2	7-9,13, 18
Y	--- WO 90 11353 A (RES CORP TECHNOLOGIES INC) 4 October 1990 see claims 1-27 --- -/-	17,26



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

14 January 1998

Date of mailing of the international search report

27-05-1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Gurdjian, D

## INTERNATIONAL SEARCH REPORT

Intern 1al Application No  
PCT/US 97/16603

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
------------	--	-----------------------

A	TSUJI S ET AL: "Signal sequence and DNA-mediated expression of human lysosomal alpha-galactosidase A." EUR J BIOCHEM, JUN 1 1987, 165 (2) P275-80, GERMANY, WEST, XP002052042 see the whole document ---	10-12, 14-16, 19,20, 23,25
A	WO 94 12628 A (SINAI SCHOOL MEDICINE) 9 June 1994 see the whole document ---	7-20,23, 25,26
A	BISHOP DF ET AL: "Human alpha-galactosidase A: nucleotide sequence of a cDNA clone encoding the mature enzyme." PROC NATL ACAD SCI U S A, JUL 1986, 83 (13) P4859-63, UNITED STATES, XP002052043 see the whole document -----	10-12, 14-16, 19,20, 23,25

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/ 16603

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
see annex
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See annex

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-20, 23, 25-29, 32-34

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-20, 23, 25-29 ,32-34

DNA molecules containing human alpha-galactosidase A and heterologous peptide signal , corresponding proteins , cells containing them , process of making glycosylated human alpha-galactosidase A and therapeutic composition .

2. Claims: 21, 22, 24 ,35

process of purification of human alpha-galactosidase A

3. Claims: 30-31

DNA molecules and proteins containing the human growth hormone signal peptide with a polypeptide other than the human growth hormone

Remark : Although claims 1-6 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/16603

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0307285 A	15-03-89	FR 2619719 A	03-03-89
		AU 606453 B	07-02-91
		AU 2179688 A	02-03-89
		DK 467188 A	02-03-89
		FI 884036 A	02-03-89
		JP 1165373 A	29-06-89
		PT 88372 A,B	31-07-89
-----			
WO 9011353 A	04-10-90	EP 0463109 A	02-01-92
		US 5658567 A	19-08-97
		US 5179023 A	12-01-93
-----			
WO 9412628 A	09-06-94	US 5401650 A	28-03-95
		AU 5681794 A	22-06-94
		CA 2150555 A	09-06-94
		EP 0670896 A	13-09-95
		JP 8503615 T	23-04-96
		US 5580757 A	03-12-96
-----			

**CORRECTED  
VERSION\***

**PCT**

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>C12N 9/40, 15/62, 15/56, A61K 38/47, 48/00, C12N 5/10</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 98/11206</b> <b>(43) International Publication Date:</b> 19 March 1998 (19.03.98)
<b>(21) International Application Number:</b> PCT/US97/16603 <b>(22) International Filing Date:</b> 12 September 1997 (12.09.97) <b>(30) Priority Data:</b> 08/712,614 13 September 1996 (13.09.96) US <b>(71) Applicant (for all designated States except US):</b> TRAN- SKARYOTIC THERAPIES, INC. [US/US]; 195 Albany Street, Cambridge, MA 02139 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SELDEN, Richard, F. [US/US]; 106 Bristol Road, Wellesley, MA 02181 (US). BOROWSKI, Marianne [US/US]; 47 Bartlett Parkway, Winthrop, MA 02152 (US). GILLESPIE, Frances, P. [US/US]; 47 Gleason Road, Lexington, MA 02173 (US). KINOSHITA, Carol, M. [US/US]; 400 Davis Road, Bed- ford, MA 01730 (US). TRECO, Douglas, A. [US/US]; 87 Brantwood Road, Arlington, MA 02174 (US). WILLIAMS, Melanie, D. [US/US]; 7 Malden Street, Natick, MA 01760 (US). <b>(74) Agent:</b> FRASER, Janis, K.; Fish & Richardson PC, 225 Franklin Street, Boston, MA 02110-2804 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> THERAPY FOR $\alpha$ -GALACTOSIDASE A DEFICIENCY  <b>(57) Abstract</b>  A therapeutic method whereby an individual suspected of having an $\alpha$ -galactosidase A deficiency, such as Fabry disease, is treated either with (1) human cells that have been genetically modified to overexpress and secrete human $\alpha$ -gal A, or (2) purified human $\alpha$ -gal A obtained from cultured, genetically modified human cells.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

THERAPY FOR  $\alpha$ -GALACTOSIDASE A DEFICIENCYBackground of the Invention

This invention relates to  $\alpha$ -galactosidase A and  
5 treatment for  $\alpha$ -galactosidase A deficiency.

Fabry disease is an X-linked inherited lysosomal storage disease characterized by symptoms such as severe renal impairment, angiokeratomas, and cardiovascular abnormalities, including ventricular enlargement and  
10 mitral valve insufficiency. The disease also affects the peripheral nervous system, causing episodes of agonizing, burning pain in the extremities. Fabry disease is caused by a deficiency in the enzyme  $\alpha$ -galactosidase A ( $\alpha$ -gal A), which results in a blockage of the catabolism of  
15 neutral glycosphingolipids, and accumulation of the enzyme's substrate, ceramide trihexoside, within cells and in the bloodstream.

Due to the X-linked inheritance pattern of the disease, essentially all Fabry disease patients are male.  
20 Although a few severely affected female heterozygotes have been observed, female heterozygotes are generally either asymptomatic or have relatively mild symptoms largely limited to a characteristic opacity of the cornea. An atypical variant of Fabry disease, exhibiting  
25 low residual  $\alpha$ -gal A activity and either very mild symptoms or apparently no other symptoms characteristic of Fabry disease, correlates with left ventricular hypertrophy and cardiac disease (Nakano et al., New Engl. J. Med. 333:288-293, 1995). It has been speculated that  
30 reduction in  $\alpha$ -gal A may be the cause of such cardiac abnormalities.

The cDNA and gene encoding human  $\alpha$ -gal A have been isolated and sequenced (Bishop et al., Proc. Natl. Acad. Sci. USA 83:4859, 1986; Kornreich et al., Nuc. Acids Res.  
35 17:3301, 1988; Oeltjen et al., Mammalian Genome 6:335-

- 2 -

338, 1995). Human  $\alpha$ -gal A is expressed as a 429-amino acid polypeptide, of which the N-terminal 31 amino acids constitute a signal peptide. The human enzyme has been expressed in Chinese Hamster Ovary (CHO) cells (Desnick, 5 U.S. Patent No. 5,356,804; Ioannou et al., J. Cell Biol. 119:1137, 1992); insect cells (Calhoun et al., U.S. Patent No. 5,179,023); and COS cells (Tsuji et al., Eur. J. Biochem. 165:275, 1987). Pilot trials of  $\alpha$ -gal A replacement therapies have been reported, using protein 10 derived from human tissues (Mapes et al., Science 169:987 1970; Brady et al., N. Engl. J. Med. 289:9, 1973; Desnick et al., Proc. Natl. Acad. Sci. USA 76:5326, 1979), but there is currently no effective treatment for Fabry disease.

15                                    Summary of the Invention

It has been found that expressing a DNA encoding human  $\alpha$ -gal A in cultured human cells produces a polypeptide that is glycosylated appropriately, so that it is not only enzymatically active and capable of acting 20 on the glycosphingolipid substrate which accumulates in Fabry disease, but is also efficiently internalized by cells via cell surface receptors which target it exactly to where it is needed in this disease: the lysosomal compartment of affected cells, particularly the 25 endothelial cells lining the patient's blood vessels. This discovery, which is discussed in more detail below, means that an individual suspected of having an  $\alpha$ -gal A deficiency such as Fabry disease can be treated either with (1) human cells that have been genetically modified 30 to overexpress and secrete human  $\alpha$ -gal A, or (2) purified human  $\alpha$ -gal A obtained from cultured, genetically modified human cells.

Therapy via the first route, i.e., with the modified cells themselves, involves genetic manipulation

- 3 -

of human cells (e.g., primary cells, secondary cells, or immortalized cells) *in vitro* or *ex vivo* to induce them to express and secrete high levels of human  $\alpha$ -gal A, followed by implantation of the cells into the patient, as generally described in Selden et al., WO 93/09222 (herein incorporated by reference).

When cells are to be genetically modified for the purposes of treatment of Fabry disease by either gene therapy or enzyme replacement therapy, a DNA molecule that contains an  $\alpha$ -gal A cDNA or genomic DNA sequence may be contained within an expression construct and introduced into primary or secondary human cells (e.g., fibroblasts, epithelial cells including mammary and intestinal epithelial cells, endothelial cells, formed elements of the blood including lymphocytes and bone marrow cells, glial cells, hepatocytes, keratinocytes, muscle cells, neural cells, or the precursors of these cell types) by standard methods of transfection including, but not limited to, liposome-, polybrene-, or DEAE dextran-mediated transfection, electroporation, calcium phosphate precipitation, microinjection, or velocity driven microprojectiles ("biolistics"). Alternatively, one could use a system that delivers DNA by viral vector. Viruses known to be useful for gene transfer include adenoviruses, adeno associated virus, herpes virus, mumps virus, poliovirus, retroviruses, Sindbis virus, and vaccinia virus such as canary pox virus. Although primary or secondary cell cultures are preferred for the therapy methods of the invention, one can also use immortalized human cells. Examples of immortalized human cell lines useful in the present methods include, but are not limited to, Bowes Melanoma cells (ATCC Accession No. CRL 9607), Daudi cells (ATCC Accession No. CCL 213), HeLa cells and derivatives of HeLa cells (ATCC Accession Nos. CCL 2, CCL 2.1, and

- 4 -

CCL 2.2), HL-60 cells (ATCC Accession No. CCL 240), HT1080 cells (ATCC Accession No. CCL 121), Jurkat cells (ATCC Accession No. TIB 152), KB carcinoma cells (ATCC Accession No. CCL 17), K-562 leukemia cells (ATCC Accession No. CCL 243), MCF-7 breast cancer cells (ATCC Accession No. BTH 22), MOLT-4 cells (ATCC Accession No. 1582), Namalwa cells (ATCC Accession No. CRL 1432), Raji cells (ATCC Accession No. CCL 86), RPMI 8226 cells (ATCC Accession No. CCL 155), U-937 cells (ATCC Accession No. CRL 1593), WI-38VA13 subline 2R4 cells (ATCC Accession No. CLL 75.1), and 2780AD ovarian carcinoma cells (Van der Blick et al., Cancer Res. 48:5927-5932, 1988) as well as heterohybridoma cells produced by fusion of human cells and cells of another species. Secondary human fibroblast strains, such as WI-38 (ATCC Accession No. CCL 75) and MRC-5 (ATCC Accession No. CCL 171), may also be used.

Following the genetic engineering of human cells with a DNA molecule encoding  $\alpha$ -gal A (or following another appropriate genetic modification, as described below) to produce a cell which overexpresses and secretes  $\alpha$ -gal A, a clonal cell strain consisting essentially of a plurality of genetically identical cultured primary human cells, or, where the cells are immortalized, a clonal cell line consisting essentially of a plurality of genetically identical immortalized human cells, may be generated. Preferably, the cells of the clonal cell strain or clonal cell line are fibroblasts.

The genetically modified cells can then be prepared and introduced into the patient by appropriate methods, e.g. as described in Selden et al., WO 93/09222.

Gene therapy in accordance with the invention possesses a number of advantages over enzyme replacement therapy with enzyme derived from human or animal tissues. For example, the method of the invention does not depend

- 5 -

upon the possibly inconsistent availability of sources of appropriate tissues, and so is a commercially viable means of treating  $\alpha$ -gal A deficiency. It is relatively risk-free compared to enzyme-replacement therapy with enzyme derived from human tissues, which may be infected with known or unknown viruses and other infective agents. Furthermore, gene therapy in accordance with the invention possesses a number of advantages over enzyme replacement therapy in general. For example, the method of the invention (1) provides the benefits of a long-term treatment strategy that eliminates the need for daily injections; (2) eliminates the extreme fluctuations in serum and tissue concentrations of the therapeutic protein, which typically accompany conventional pharmacologic delivery; and (3) is likely to be less expensive than enzyme replacement therapy because production and purification of the protein for frequent administration are unnecessary.

As described above, individuals with  $\alpha$ -gal A deficiencies may also be treated with purified  $\alpha$ -gal A (i.e. enzyme replacement therapy). Primary, secondary, or immortalized human cells genetically modified to overexpress human  $\alpha$ -gal A will also be useful for *in vitro* protein production, to produce protein which may be purified for enzyme replacement therapy. Secondary or immortalized human cells may be chosen from among those described above and may be genetically modified by the transfection or transduction methods also described above. After genetic modification, the cells are cultured under conditions permitting overexpression and secretion of  $\alpha$ -gal A. The protein is isolated from the cultured cells by collecting the medium in which the cells are grown, and/or lysing the cells to release their contents, and then applying standard protein purification techniques. One such technique involves passing the



- 6 -

culture medium, or any sample containing human  $\alpha$ -gal A, over a hydrophobic interaction resin such as Butyl Sepharose® or another resin having a functional moiety that includes a butyl group. Passing the sample over  
5 such a resin may constitute the first chromatography step. If further purification is required, the  $\alpha$ -gal A-containing material eluted from the hydrophobic interaction resin may be passed over a column containing a second resin, such as an immobilized heparin resin such  
10 as Heparin Sepharose®, hydroxyapatite, an anion exchange resin such as Q Sepharose®, or a size exclusion resin such as Superdex® 200. Preferably, the purification protocol would include use of each of the above types of resins. Alternatively, one could use one or more of the  
15 latter resins before or instead of the hydrophobic interaction resin.

Previous methods for the preparation of  $\alpha$ -gal A with relatively high purity were dependent on the use of affinity chromatography, using a combination of lectin  
20 affinity chromatography (concanavalin A (Con A) Sepharose) and affinity chromatography based on binding of  $\alpha$ -gal A to the substrate analog N-6-aminohexanoyl- $\alpha$ -D-galactosylamine coupled to a Sepharose matrix (Bishop et al., J. Biol. Chem. 256:1307-1316, 1981). The use of  
25 proteinaceous lectin affinity resins and substrate analog resins is typically associated with the continuous leaching of the affinity agent from the solid support (cf. Marikar et al., Anal. Biochem. 201:306-310, 1992), resulting in contamination of the purified product with  
30 the affinity agent either free in solution or bound to eluted protein. Such contaminants make the product unsuitable for use in pharmaceutical preparations. Bound substrate analogs and lectins can also have substantial negative effects on the enzymatic, functional, and  
35 structural properties of proteins. Furthermore, such

- 7 -

affinity resins are typically expensive to prepare, making the use of such resins less suitable for production on a commercial scale than more conventional chromatography resins. Thus, the development of a purification protocol using conventional chromatography resins, which are readily available in supplies and quality suitable for large-scale commercial use, is a significant advantage of the present invention.

An individual who is suspected of having an  $\alpha$ -gal A deficiency may be treated by administration of pharmaceutically acceptable, purified human  $\alpha$ -gal A by any standard method, including but not limited to intravenous, subcutaneous, or intramuscular injection, or as a solid implant. The purified protein may be formulated in a therapeutic composition consisting of an aqueous solution containing a physiologically acceptable excipient, e.g. a carrier such as human serum albumin, at pH 6.5 or below.

The present invention thus provides a means for obtaining large quantities of appropriately glycosylated and therefore therapeutically useful human  $\alpha$ -gal A. This makes enzyme replacement therapy for  $\alpha$ -gal deficiency commercially viable, as well as relatively risk-free compared to therapy with enzyme derived from human or animal tissues.

Skilled artisans will recognize that the human  $\alpha$ -gal A DNA sequence (either cDNA or genomic DNA), or sequences that differ from it due to either silent codon changes or to codon changes that produce conservative amino acid substitutions, can be used to genetically modify cultured human cells so that they will overexpress and secrete the enzyme. It is also possible that certain mutations in the  $\alpha$ -gal A DNA sequence will encode polypeptides that retain or exhibit improved  $\alpha$ -gal A enzymatic activity (as would be apparent by expressing

- 8 -

the mutant DNA molecule in cultured cells, purifying the encoded polypeptide, and measuring the catalytic activity, as described herein). For example, one would expect conservative amino acid substitutions to have  
5 little or no effect on the biological activity, particularly if they represent less than 10% of the total number of residues in the protein. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine,  
10 leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

Production of  $\alpha$ -gal A by the cells can be maximized by certain genetic manipulations. For example,  
15 the DNA molecule that encodes  $\alpha$ -gal A may also encode an heterologous signal peptide, such as the signal peptide of human growth hormone (hGH), erythropoietin, Factor VIII, Factor IX, glucagon, the low density lipoprotein (LDL) receptor, or a lysosomal enzyme other  
20 than  $\alpha$ -gal A. Preferably, the signal peptide is the hGH signal peptide (SEQ ID NO:21), and is at the N-terminus of the encoded protein. The DNA sequence encoding the signal peptide may contain an intron such as the first intron of the hGH gene, resulting in a DNA sequence  
25 such as SEQ ID NO:27 (see also Fig. 10). Furthermore, the DNA molecule may also contain a 3' untranslated sequence (UTS) that is at least 6 nucleotides in length (in contrast to the  $\alpha$ -gal A mRNA found in humans, which has no 3' UTS, the requisite polyadenylation site being  
30 within the coding sequence). The UTS is positioned immediately 3' to the termination codon of the coding sequence, and includes a polyadenylation site. It is preferably at least 6 nucleotides in length, more preferably at least 12, and most preferably at least 30,  
35 and in all cases it contains the sequence AATAAA or a

- 9 -

related sequence which serves to promote polyadenylation. A DNA molecule as described, i.e., encoding an hGH signal peptide linked to  $\alpha$ -gal A and containing a 3' UTS that includes a polyadenylation site, and preferably including  
5 expression control sequences, is also within the invention. Also within the scope of the invention is a DNA molecule encoding a protein that includes the signal peptide of hGH linked to  $\alpha$ -gal A or any other heterologous polypeptide (i.e., any polypeptide other  
10 than hGH or an analog of hGH). The heterologous polypeptide is typically a mammalian protein, e.g. any medically desirable human polypeptide.

Other features and advantages of the invention will be apparent from the detailed description that  
15 follows, and from the claims.

The term "genetically modified," as used herein in reference to cells, is meant to encompass cells that express a particular gene product following introduction of a DNA molecule encoding the gene product and/or  
20 regulatory elements that control expression of a coding sequence. The introduction of the DNA molecule may be accomplished by gene targeting (i.e., introduction of a DNA molecule to a particular genomic site); furthermore homologous recombination allows replacement of the  
25 defective gene itself (the defective  $\alpha$ -gal A gene or a portion of it could be replaced in a Fabry disease patient's own cells with the whole gene or a portion thereof)).

The term " $\alpha$ -gal A," as used herein, means  $\alpha$ -gal A  
30 without a signal peptide, i.e., SEQ ID NO:26 (Fig. 9). There is some indication that residues 371 to 398 or 373 to 398 of SEQ ID NO:26 (Fig. 9) may be removed in the lysosome; however, removal of this putative propeptide is not believed to affect activity of the enzyme. This  
35 suggests that any portion of the putative propeptide

- 10 -

could be deleted without affecting activity. Thus, the term " $\alpha$ -gal A" as used herein also covers a protein having a sequence corresponding to SEQ ID NO:26 except lacking up to 28 residues at the C-terminus of that  
5 sequence.

By " $\alpha$ -gal A deficiency" is meant any deficiency in the amount or activity of this enzyme in a patient. The deficiency may induce severe symptoms as typically observed in males who are suffering from Fabry disease,  
10 or may be only partial and induce relatively mild symptoms as can be seen in heterozygous female carriers of the defective gene.

As used herein, the term "primary cell" includes cells present in a suspension of cells isolated from a  
15 vertebrate tissue source (prior to their being plated, i.e., attached to a tissue culture substrate such as a dish or flask), cells present in an explant derived from tissue, both of the previous types of cells plated for the first time, and cell suspensions derived from these  
20 plated cells.

"Secondary cells" refers to cells at all subsequent steps in culturing. That is, the first time a plated primary cell is removed from the culture substrate and replated (passaged), it is referred to as a secondary  
25 cell, as are all cells in subsequent passages.

A "cell strain" consists of secondary cells which have been passaged one or more times; exhibit a finite number of mean population doublings in culture; exhibit the properties of contact-inhibited, anchorage dependent  
30 growth (except for cells propagated in suspension culture); and are not immortalized.

By "immortalized cell" is meant a cell from an established cell line that exhibits an apparently unlimited lifespan in culture.

- 11 -

By "signal peptide" is meant a peptide sequence that directs a newly synthesized polypeptide to which it is attached to the endoplasmic reticulum for further post-translational processing and/or distribution.

5       The term "heterologous signal peptide," as used herein in the context of  $\alpha$ -gal A, means a signal peptide that is not the human  $\alpha$ -gal A signal peptide (i.e., that encoded by nucleotides 36-128 of SEQ ID NO:18). It typically is the signal peptide of some mammalian protein  
10 other than  $\alpha$ -gal A.

The term "first chromatography step" refers to the first application of a sample to a chromatography column (all steps associated with the preparation of the sample are excluded).

15       Brief Description of the Drawings

Fig. 1 is a representation of the 210 bp probe that was used to isolate  $\alpha$ -gal A from a human fibroblast cDNA library (SEQ ID NO:19). The sequence is from exon 7 of the  $\alpha$ -gal A gene. The probe was isolated from human  
20 genomic DNA by the polymerase chain reaction (PCR). The regions underlined in the figure correspond to the sequences of the amplification primers.

Fig. 2 is a representation of the sequence of the DNA fragment that completes the 5' end of the  $\alpha$ -gal A  
25 cDNA clone (SEQ ID NO:20). This fragment was amplified from human genomic DNA by PCR. The regions underlined correspond to the sequences of the amplification primers. The positions of the NcoI and SacII restriction endonuclease sites, which were used for subcloning as  
30 described in Example IA, are also shown.

Fig. 3 is a representation of the sequence of  $\alpha$ -gal A cDNA, including the sequence that encodes the signal peptide (SEQ ID NO:18).

Fig. 4 is a schematic map of pXAG-16, an  $\alpha$ -gal A  
35 expression construct that includes the

- 12 -

CMV (cytomegalovirus) promoter and intron, the hGH signal peptide coding sequence and first intron, the cDNA for  $\alpha$ -gal A (i.e., lacking the  $\alpha$ -gal A signal peptide sequence) and the hGH 3' UTS.

5            Fig. 5 is a schematic map of pXAG-28, an  $\alpha$ -gal A expression construct that includes the collagen I $\alpha$ 2 promoter, a  $\beta$ -actin intron, the hGH signal peptide coding sequence and first intron, the cDNA for  $\alpha$ -gal A (i.e., lacking the  $\alpha$ -gal A signal peptide sequence) and the hGH  
10 3' UTS.

Fig. 6 is a chromatogram of the  $\alpha$ -gal A purification step using Butyl Sepharose® resin. The absorbance at 280 nm (plain line) and  $\alpha$ -gal A activity (dotted line) of selected fractions is shown.

15            Fig. 7 is a line graph depicting internalization by Fabry fibroblasts of human  $\alpha$ -gal A prepared in accordance with the invention. The intracellular  $\alpha$ -gal A activity and total protein concentration were measured after incubation of the cells with increasing  
20 concentrations of human  $\alpha$ -gal A prepared in accordance with the invention. The effects of the potential internalization-inhibitors mannose-6 phosphate (M6P; open diamonds) and mannan (open circles) are shown.

Fig. 8 is a schematic diagram of the experimental  
25 paradigm designed to examine Fabry fibroblasts following internalization of  $\alpha$ -gal A. The  $\alpha$ -gal A activity of the Fabry cells is measured after exposure to either normal or  $\alpha$ -gal A-overexpressing human fibroblasts cultured in Transwell™ inserts. "M6P" = mannose-6 phosphate;  
30 "Untrf HF" = untransfected human fibroblasts; "BRS11" = a transfected,  $\alpha$ -gal A-overexpressing fibroblast strain.

Fig. 9 is a representation of the human  $\alpha$ -gal A amino acid sequence (SEQ ID NO:26).

- 13 -

Fig. 10 is a representation of the DNA sequence encoding the hGH signal peptide and containing the first hGH intron (underlined) (SEQ ID NO:27).

Fig. 11 is a representation of the DNA sequence  
5 encoding the hGH signal peptide without the intron (SEQ ID NO:22).

Fig. 12 is a representation of the amino acid sequence of the hGH signal peptide (SEQ ID NO:21).

Fig. 13 is a representation of the cDNA sequence  
10 encoding human  $\alpha$ -gal A (without signal peptide) (SEQ ID NO:25).

#### Detailed Description

Lysosomal enzymes such as  $\alpha$ -gal A are targeted to the lysosomal compartment of a cell through interaction  
15 with the mannose-6-phosphate (M6P) receptor, which binds to M6P residues present in the oligosaccharide moieties of enzymes destined for the lysosomal compartment (Kornfeld, S. and Mellman, I, Ann. Rev. Cell Biol. 5:483-525, 1989). The primary interaction occurs in the Golgi,  
20 where enzymes bound to Golgi M6P receptors are segregated for transport to the lysosomes. A secondary type of interaction is believed to take place between extracellular  $\alpha$ -gal A and M6P receptors at the cell surface. Extracellular substances internalized by cells  
25 are transported through the cytoplasm in endocytic vesicles, which fuse with primary lysosomes and empty their contents into the lysosomes. In this process, cell surface M6P receptors are also incorporated into endocytic vesicles and transported to lysosomes.

30 Any  $\alpha$ -gal A present in the extracellular milieu can, if it bears M6P residues, bind to the cell surface M6P receptors and thereby be transported into the lysosomal compartment along with the receptors. Once in the lysosomal compartment as a result of this scavenger



- 14 -

pathway, the enzyme can carry out its appropriate function. Thus, even if a cell is genetically deficient in producing its own  $\alpha$ -gal A, there exists a mechanism for it to take up exogenously produced enzyme, provided  
5 that (a) the enzyme is suitably glycosylated and (b) the deficient cell bears M6P receptors.

In Fabry disease, vascular endothelial cells of the kidney and heart have been shown to display severe histopathologic abnormalities and contribute to the  
10 clinical pathology of the disease; these cells, which do carry M6P receptors, are a particular target of the presently claimed invention. The  $\alpha$ -gal A produced in accordance with the invention may be delivered either locally or systemically to the affected cells, by gene  
15 therapy (i.e., by genetically modified cells which express and secrete the glycosylated enzyme within the patient), or by conventional pharmacologic routes of administration. An  $\alpha$ -gal A in which M6P is present in the N-linked oligosaccharides is therefore of great  
20 importance for therapy in accordance with the invention.

Furthermore, the degree to which the N-linked oligosaccharides of  $\alpha$ -gal A are modified by sialylation is also of great importance. In the absence of appropriate sialylation,  $\alpha$ -gal A will be rapidly cleared  
25 from the circulation due to binding by hepatic asialoglycoprotein receptors, followed by internalization and degradation by hepatocytes (Ashwell and Harford, Ann. Rev. Biochem. 51:531-554, 1982). This decreases the amount of  $\alpha$ -gal A available in the circulation for  
30 binding to M6P receptors on cells which contribute to the clinical pathology of Fabry disease, such as the vascular endothelial cells of the kidney and heart. Surprisingly, the Applicants have found that  $\alpha$ -gal A secreted by stably transfected human cells has glycosylation properties  
35 which are suitable for the treatment of Fabry disease by

- 15 -

either gene therapy or by conventional pharmaceutical administration of the purified secreted protein. This is in contrast to the situation with the best-studied lysosomal enzyme, glucocerebrosidase, in which delivery of the enzyme purified from human placenta or secreted from transfected CHO cells to the clinically-relevant cells in the body requires complex enzymatic modification of the enzyme following purification (cf. Beutler, New Engl. J. Med. 325:1354-1360, 1991).

10       The therapy of the invention can be carried out in either of two general ways: by introducing into the patient a therapeutically effective amount of purified human  $\alpha$ -gal A obtained from cultured human cells genetically modified to overexpress and secrete the enzyme, or by introducing the overexpressing cell itself into the patient. Techniques to accomplish the necessary genetic modifications are discussed below, as are the methods of purification, formulation, and treatment.

Example I. Preparation and Use of Constructs Designed to Deliver and Express  $\alpha$ -Gal A

20       Two expression plasmids, pXAG-16 and pXAG-28, were constructed. These plasmids contain human  $\alpha$ -gal A cDNA encoding the 398 amino acids of the  $\alpha$ -gal A enzyme (without the  $\alpha$ -gal A signal peptide); the human growth hormone (hGH) signal peptide genomic DNA sequence, which is interrupted by the first intron of the hGH gene; and the 3' untranslated sequence (UTS) of the hGH gene, which contains a signal for polyadenylation. Plasmid pXAG-16 has the human cytomegalovirus immediate-early (CMV IE) promoter and first intron (flanked by non-coding exon sequences), while pXAG-28 is driven by the collagen I $\alpha$ 2 promoter and also contains the  $\beta$ -actin gene's 5' UTS, which contains the first intron of the  $\beta$ -actin gene.

- 16 -

A. Cloning of the Complete  $\alpha$ -Gal A  
cDNA, and Construction of the  $\alpha$ -Gal A  
Expression Plasmid pXAG-16

The human  $\alpha$ -gal cDNA was cloned from a human  
5 fibroblast cDNA library that was constructed as follows.  
Poly-A<sup>+</sup> mRNA was isolated from total RNA, and cDNA  
synthesis was performed using reagents for the  
lambda ZapII® system according to the manufacturer's  
instructions (Stratagene Inc., LaJolla, CA). Briefly,  
10 "first strand" cDNA was generated by reverse  
transcription in the presence of an oligo-dT primer  
containing an internal XhoI restriction endonuclease  
site. Following treatment with RNase H, the cDNA was  
nick-translated with DNA polymerase I to generate double  
15 stranded cDNA. This cDNA was made blunt-ended with  
T4 DNA polymerase, and ligated to EcoRI adaptors. The  
products of this ligation were treated with T4 DNA kinase  
and digested with XhoI. The cDNA was fractionated by  
Sephacryl-400® chromatography. Large and medium size  
20 fractions were pooled and the cDNAs ligated to EcoRI and  
XhoI-digested Lambda ZapII arms. The products of this  
ligation were then packaged and titered. The primary  
library had a titer of  $1.2 \times 10^7$  pfu/ml and an average  
insert size of 925 bp.

25 A 210 bp probe from exon 7 of the human  $\alpha$ -gal A  
gene (Fig. 1, SEQ ID NO:19) was used to isolate the cDNA.  
The probe itself was isolated from genomic DNA by the  
polymerase chain reaction (PCR) using the following  
oligonucleotides: 5'-CTGGGCTGTAGCTATGATAAAC-3' (Oligo 1;  
30 SEQ ID NO:1) and 5'-TCTAGCTGAAGCAAAACAGTG-3' (Oligo 2;  
SEQ ID NO:2). The PCR product was then used to screen  
the fibroblast cDNA library, and positive clones were  
isolated and further characterized. One positive clone,  
phage 3A, was subjected to the lambda ZapII® system  
35 excision protocol (Stratagene, Inc., La Jolla, CA),

- 17 -

according to the manufacturer's instructions. This procedure yielded plasmid pBSAG3A, which contains the  $\alpha$ -gal A cDNA sequence in the pBluescriptSK-<sup>™</sup> plasmid backbone. DNA sequencing revealed that this plasmid did not contain the complete 5' end of the cDNA sequence. Therefore, the 5' end was reconstructed using a PCR fragment amplified from human genomic DNA. To accomplish this, a 268 bp genomic DNA fragment (Fig. 2, SEQ ID NO:20) was amplified using the following

10 oligonucleotides: 5'-ATTGGTCCGCCCCTGAGGT-3' (Oligo 3; SEQ ID NO:3) and 5'-TGATGCAGGAATCTGGCTCT-3' (Oligo 4; SEQ ID NO:4). This fragment was subcloned into a "TA" cloning plasmid (Invitrogen Corp., San Diego, CA) to generate plasmid pTAAGEI. Plasmid pBSAG3A, which

15 contains the majority of the  $\alpha$ -gal A cDNA sequence, and pTAAGEI, which contains the 5' end of the  $\alpha$ -gal A cDNA, were each digested with SacII and NcoI. The positions of the relevant SacII and NcoI sites within the amplified DNA fragment are shown in Fig. 2. The 0.2 kb SacII-NcoI

20 fragment from pTAAGEI was isolated and ligated to equivalently digested pBSAG3A. This plasmid, pAGAL, contains the complete  $\alpha$ -gal A cDNA sequence, including the sequence encoding the  $\alpha$ -gal A signal peptide. The cDNA was completely sequenced (shown in Fig. 3 including

25 the  $\alpha$ -gal A signal peptide; SEQ ID NO: 18) and found to be identical to the published sequence for the human  $\alpha$ -gal A cDNA (Genbank sequence HUMGALA).

The plasmid pXAG-16 was constructed via several intermediates, as follows. First, pAGAL was digested

30 with SacII and XhoI and blunt-ended. Second, the ends of the complete  $\alpha$ -gal A cDNA were ligated to XbaI linkers and subcloned into XbaI digested pEF-BOS (Mizushima et al., Nucl. Acids Res. 18:5322, 1990), creating pXAG-1. This construct contains the human granulocyte-colony

35 stimulating factor (G-CSF) 3' UTS and the human

- 18 -

elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) promoter flanking the cDNA encoding  $\alpha$ -gal A plus the  $\alpha$ -gal A signal peptide, such that the 5' end of the  $\alpha$ -gal A cDNA is fused to the EF-1 $\alpha$  promoter. To create a construct with the CMV IE promoter and first intron, the  $\alpha$ -gal A cDNA and G-CSF 3' UTS were removed from pXAG-1 as a two kb XbaI-BamHI fragment. The fragment was blunt-ended, ligated to BamHI linkers, and inserted into BamHI digested pCMVflpNeo (which was constructed as described below). The orientation was such that the 5' end of the  $\alpha$ -gal A cDNA was fused to the CMV IE promoter region.

pCMVflpNeo was created as follows. A CMV IE gene promoter fragment was amplified by PCR using CMV genomic DNA as a template and the oligonucleotides:

5'-TTTTGGATCCCTCGAGGACATTGATTATTGACTAG-3' (SEQ ID NO:23)  
and 5'-TTTTGGATCCCGTGTCAAGGACGGTGAC-3' (SEQ ID NO:24).

The resulting product (a 1.6 kb fragment) was digested with BamHI, yielding a CMV promoter-containing fragment with cohesive BamHI-digested ends. The neo expression unit was isolated from plasmid pMC1neoPA (Stratagene Inc., La Jolla, CA) as a 1.1 kb XhoI-BamHI fragment. The CMV promoter-containing and neo fragments were inserted into a BamHI-, XhoI-digested plasmid (pUC12). Notably, pCMVflpNeo contains the CMV IE promoter region, beginning at nucleotide 546 and ending at nucleotide 2105 (of Genbank sequence HS5MIEP), and the neomycin resistance gene driven by the Herpes Simplex Virus (HSV) thymidine kinase promoter (the TKneo gene) immediately 5' to the CMV IE promoter fragment. The direction of transcription of the neo gene is the same as that of the CMV promoter fragment. This intermediate construct was called pXAG-4.

To add the hGH 3' UTS, the GCSF 3' UTS was removed from pXAG-4 as an XbaI-SmaI fragment and the ends of pXAG-4 were made blunt. The hGH 3' UTS was removed from pXGH5 (Selden et al., Mol. Cellular Biol. 6:3173-3179,

- 19 -

1986) as a 0.6 kb SmaI-EcoRI fragment. After blunt-ending this fragment, it was ligated into pXAG-4 immediately after the blunt-ended XbaI site of pXAG-4. This intermediate was called pXAG-7. The TKneo fragment  
5 was removed from this plasmid as a HindIII-ClaI fragment and the ends of the plasmid were blunted by "filling-in" with the Klenow fragment of DNA polymerase I. A neomycin resistance gene driven by the SV40 early promoter was ligated in as a blunted ClaI-BsmBI fragment from a digest  
10 of pcDNeo (Chen et al., Mol. Cellular Biol. 7:2745-2752, 1987), placing the neo transcription unit in the same orientation as the  $\alpha$ -gal A transcription unit. This intermediate was called pXAG-13.

To complete pXAG-16, which has the 26 amino acid  
15 hGH signal peptide coding sequence and first intron of the hGH gene, a 2.0 kb EcoRI-BamHI fragment of pXAG-13 was first removed. This fragment included the  $\alpha$ -gal A cDNA and the hGH 3' UTS. This large fragment was replaced with 3 fragments. The first fragment consisted  
20 of a 0.3 kb PCR product of pXGH5, which contains the hGH signal peptide coding sequence and includes the hGH first intron sequence, from a synthetic BamHI site located just upstream of the Kozak consensus sequence to the end of the hGH signal peptide coding sequence. The following  
25 oligonucleotides were used to amplify this fragment (Fragment 1): 5'-TTTTGGATCCACCATGGCTA-3' (Oligo HGH101; SEQ ID NO:5) and 5'-TTTTGCCGGCACTGCCCTCTTGAA-3' (Oligo HGH102; SEQ ID NO:6). The second fragment consisted of a 0.27 kb PCR product containing sequences  
30 corresponding to the start of the cDNA encoding the 398 amino acid  $\alpha$ -gal A enzyme (i.e., lacking the  $\alpha$ -gal A signal peptide) to the NheI site. The following oligonucleotides were used to amplify this fragment (Fragment 2): 5'-TTTTCAGCTGGACAATGGATTGGC-3' (Oligo AG10;  
35 SEQ ID NO:7) and 5'-TTTTGCTAGCTGGCGAATCC-3' (Oligo AG11;

- 20 -

SEQ ID NO:8). The third fragment consisted of the NheI-EcoRI fragment of pXAG-7 containing the remaining  $\alpha$ -gal A sequence as well as the hGH 3' UTS (Fragment 3).

Fragment 1 (digested with BamHI and NaeI),  
5 Fragment 2 (digested with PvuII and NheI), and Fragment 3 were mixed with the 6.5 kb BamHI-EcoRI fragment of pXAG-13 containing the neo gene and the CMV IE promoter and ligated together to generate plasmid pXAG-16 (Figure 4).

#### B. Construction of the $\alpha$ -Gal A

##### 10 Expression Plasmid pXAG-28

The human collagen Ia2 promoter was isolated for use in the  $\alpha$ -gal A expression construct pXAG-28 as follows. A 408 bp PCR fragment of human genomic DNA containing part of the human collagen Ia2 promoter was  
15 isolated using the following oligonucleotides:  
5'-TTTTGGATCCGTGTCCCATAGTGTTCCTCAA-3' (Oligo 72;  
SEQ ID NO:9) and 5'-TTTTGGATCCGTCAGTCGTGGCCAGTACC-3'  
(Oligo 73; SEQ ID NO:10).

This fragment was used to screen a human leukocyte  
20 library in EMBL3 (Clontech Inc., Palo Alto, CA). One positive clone (phage 7H) containing a 3.8 kb EcoRI fragment was isolated and cloned into pBSIISK+ (Stratagene Inc., La Jolla, CA) at the EcoRI site (creating pBS/7H.2). An AvrII site was introduced in  
25 pBSIISK+ by digesting with SpeI, which cleaves within the pBSIISK+ polylinker, "filling-in" with the Klenow fragment of DNA polymerase I, and inserting the oligonucleotide 5'-CTAGTCCTAGGA-3' (SEQ ID NO:11). This variant of pBSIISK+ was digested with BamHI and AvrII and  
30 ligated to the 121 bp BamHI-AvrII fragment of the original 408 bp collagen Ia2 promoter PCR fragment described above, creating pBS/121COL.6.

The plasmid pBS/121COL.6 was digested with XbaI,  
which cleaves within the pBSIISK+ polylinker sequence,  
35 "filled-in" with the Klenow fragment of DNA polymerase I,

- 21 -

and digested with AvrII. The 3.8 kb BamHI-AvrII fragment of pBS/7H.2 was isolated and the BamHI site made blunt-ended by treatment with Klenow enzyme. The fragment was then digested with AvrII and ligated to the 5 AvrII-digested vector, thus creating the collagen promoter plasmid pBS/121bpCOL7H.18.

Next the collagen promoter was fused to the 5' UTS of the human  $\beta$ -actin gene, which contains the first intron of the human  $\beta$ -actin gene. To isolate this 10 sequence, a 2 kb PCR fragment was isolated from human genomic DNA using the following oligonucleotides:  
5'-TTTTGAGCACAGAGCCTCGCCT-3' (Oligo BA1; SEQ ID NO:12)  
and  
5'-TTTTGGATCCGGTGAGCTGCGAGAATAGCC-3' (Oligo BA2;  
15 SEQ ID NO:13).

This fragment was digested with BamHI and BsiHKAI to release a 0.8 kb fragment containing the  $\beta$ -actin 5' UTS and intron. A 3.6 kb SalI-SrfI fragment was then isolated from the collagen promoter plasmid 20 pBS/121bpCOL7H.18 as follows. pBS/121bpCOL7H.18 was partially digested with BamHI (the BamHI site lies at the 5' end of the collagen I $\alpha$ 2 promoter fragment), made blunt-ended by treatment with the Klenow fragment, and ligated to a SalI linker (5'-GGTCGACC-3'), thereby 25 placing a SalI site upstream of the collagen I $\alpha$ 2 promoter. This plasmid was then digested with SalI and SrfI (the SrfI site lies 110 bp upstream of the collagen I $\alpha$ 2 promoter CAP site), and the 3.6 kb fragment was isolated. The 0.8 and 3.6 kb fragments were combined with SalI - 30 and BamHI - digested pBSIISK- (Stratagene Inc., La Jolla, CA), and a fragment composed of the following four oligonucleotides annealed together (forming a fragment with a blunt end and a BsiHKAI end):  
5'-GGGCCCCCAGCCCCAGCCCTCCATTGGTGGAGGCCCTTTGGAGGCACCCTAG  
35 GGCCAGGAAACTTTTGCCGTAT-3' (Oligo COL-1; SEQ ID NO:14),



- 22 -

5'-AAATAGGGCAGATCCGGGCTTTATTATTTTAGCACCACGGCCGCGAGAC  
 CGCGTCCGCCCCGCGAGCA-3' (Oligo COL-2; SEQ ID NO:15),  
 5'-TGCCCTATTTATACGGCAAAAGTTTCCTGGCCCTAGGGTGCCTCCAAAAGGGC  
 CTCCACCAATGGGAGGGCTGGGGCTGGGGGCCC-3' (Oligo COL-3;  
 5 SEQ ID NO:16), and 5'-CGCGGGGCGGACGCGGTCTCGGCGGCCGTGGTGCT  
 AAAATAATAAGCCCGGATC-3' (Oligo COL-4; SEQ ID NO:17).  
 These four oligonucleotides, when annealed, correspond to  
 the region beginning at the SrfI site of the collagen  
 promoter and continuing through the BsiHKAI site of the  
 10  $\beta$ -actin promoter. The resulting plasmid was designated  
 pCOL/ $\beta$ -actin.

To complete the construction of pXAG-28, the  
 Sali-BamHI fragment of pCOL/ $\beta$ -actin, containing the  
 collagen I $\alpha$ 2 promoter and  $\beta$ -actin 5' UTS, was isolated.  
 15 This fragment was ligated to two fragments from pXAG-16  
 (see Example 1A and Fig. 4): (1) the 6.0 kb BamHI  
 fragment (containing the neo gene, plasmid backbone, the  
 cDNA encoding the 398 amino acid  $\alpha$ -gal A enzyme, and the  
 hGH 3' UTS); and (2) the 0.3 kb BamHI-XhoI fragment  
 20 (which contains the SV40 poly A sequence from pcdneo).  
 pXAG-28 contains the human collagen I $\alpha$ 2 promoter fused to  
 the human  $\beta$ -actin 5' UTS, the hGH signal peptide (which  
 is interrupted by the hGH first intron), the cDNA  
 encoding the  $\alpha$ -gal A enzyme, and the hGH 3' UTS. A map  
 25 of the completed expression construct pXAG-28 is shown in  
 Fig. 5.

### C. Transfection and Selection of Fibroblasts Electroporated with $\alpha$ -Gal A Expression Plasmids

In order to express  $\alpha$ -gal A in fibroblasts,  
 30 secondary fibroblasts were cultured and transfected  
 according to published procedures (Selden et al.,  
 WO 93/09222).

The plasmids pXAG-13, pXAG-16 and pXAG-28 were  
 transfected by electroporation into human foreskin  
 35 fibroblasts to generate stably transfected clonal cell

- 23 -

strains, and the resulting  $\alpha$ -gal A expression levels were monitored as described in Example ID. Secretion of  $\alpha$ -gal A by normal foreskin fibroblasts is in the range of 2-10 units/ $10^6$  cells/24 hours. In contrast, the 5 transfected fibroblasts displayed mean expression levels as shown in Table 1:

Table 1: Mean  $\alpha$ -gal A expression levels (+/- standard deviation)

---

10	pXAG-13:	420 +/- 344 U/ $10^6$ cells/day N=26 clonal strains (range 3 - 1133 U/ $10^6$ cells/day)
	pXAG-16:	2,051 +/- 1253 U/ $10^6$ cells/day N=24 clonal strains 15 (range 422 - 5200 U/ $10^6$ cells/day)
	pXAG-28:	141 +/- 131 U/ $10^6$ cells/day N=38 clonal strains (range 20 - 616 U/ $10^6$ cells/day)

These data show that all three expression  
20 constructs are capable of increasing  $\alpha$ -gal A expression many times that of nontransfected fibroblasts. Expression by fibroblasts stably transfected with pXAG-13, which encodes  $\alpha$ -gal A linked to the  $\alpha$ -gal A signal peptide, was substantially lower than expression by  
25 fibroblasts transfected with pXAG-16, which differs only in that the signal peptide is the hGH signal peptide, the coding sequence of which is interrupted by the first intron of the hGH gene.

- 24 -

Each time the transfected cells were passaged, the secreted  $\alpha$ -gal A activity was determined, the cells were counted, and the cell density was calculated. Based on the number of cells harvested and the time allowed for secretion of  $\alpha$ -gal A, the specific expression rate of  $\alpha$ -gal A was determined and is reported in Tables 2 and 3 as secreted units (of  $\alpha$ -gal A) per  $10^6$  cells per 24 hour period. Cell strains desirable for gene therapy or for use in generation of material for purification of  $\alpha$ -gal A should display stable growth and expression over several passages. Data from the cell strains shown in Tables 2 and 3, which were stably transfected with the  $\alpha$ -gal A expression construct pXAG-16, illustrate the fact that  $\alpha$ -gal A expression is stably maintained during serial passage.

Table 2: Growth and Expression of BRS-11 Cells Containing the  $\alpha$ -Gal A Expression Construct pXAG-16.

BRS-11		
Passage	Expression (units/ $10^6$ cells/24 hr)	Cell Density (cells/cm <sup>2</sup> )
13	2601	$4.80 \times 10^4$
14	1616	$4.40 \times 10^4$
15	3595	$4.40 \times 10^4$

- 25 -

Table 3: Growth and Expression of HF503-242 Cells  
Containing the  $\alpha$ -Gal A Expression Construct  
pXAG-16.

HF503-242		
Passage	Expression (units/ $10^6$ cells/24hr)	Cell Density (cells/cm <sup>2</sup> )
5	4069	$2.80 \times 10^4$
6	7585	$3.55 \times 10^4$
7	5034	$2.48 \times 10^4$

#### D. Quantification of $\alpha$ -Gal A Expression

The activity of  $\alpha$ -gal A activity was measured using the water-soluble substrate 4-methylumbelliferyl- $\alpha$ -D-galactopyranoside (4-MUF-gal; Research Products, Inc.) by a modification of the protocol described by Ioannou et al. (J. Cell Biol. 119:1137-1150, 1992). The substrate was dissolved in substrate buffer (0.1 M citrate-phosphate, pH 4.6) to a concentration of 1.69 mg/ml (5 mM). Typically, 10  $\mu$ l of culture supernatant was added to 75  $\mu$ l of the substrate solution. The tubes were covered and allowed to incubate in a 37°C waterbath for 60 minutes. At the end of the incubation period, 2 ml of glycine-carbonate buffer (130 mM glycine, 83 mM sodium carbonate, at pH 10.6), were used to stop the reaction. The relative fluorescence of each sample was measured using a model TK0100 fluorometer (Hoefer Scientific Instruments) which has a fixed excitation wavelength of 365 nm and detects a fixed emission wavelength of 460 nm. The

- 26 -

readings of the samples were compared to standards prepared from a 1  $\mu$ M stock of methylumbelliferone (Sigma Chemical Co.), and the amount of hydrolyzed substrate was calculated. The activity of  $\alpha$ -gal A is expressed in units; one unit of  $\alpha$ -gal A activity is equivalent to one nanomole of substrate hydrolyzed per hour at 37°C. Cell expression data were generally expressed as units of  $\alpha$ -gal A activity secreted/ $10^6$  cells/24 hours. This assay was also used to measure the amount of  $\alpha$ -gal activity in cell lysates and in samples from various  $\alpha$ -gal purification steps, as discussed below.

Example II. Purification of  $\alpha$ -Gal A from the Conditioned Medium of Stably Transfected Human Cell Strains

Examples IIA-IIE illustrate that  $\alpha$ -gal A may be purified to near-homogeneity from the conditioned medium of cultured human cell strains that have been stably transfected to produce the enzyme.

A. Use of Butyl Sepharose<sup>®</sup> Chromatography as a First Step in the Purification of  $\alpha$ -Gal A

Cold conditioned medium (1.34 liters) was clarified by centrifugation and filtered through a 0.45  $\mu$ m cellulose acetate filter using glass fiber prefilters. While stirring, the pH of the cold, filtered medium was adjusted to 5.6 by the dropwise addition of 1 N HCl, and ammonium sulfate was added to a final concentration of 0.66 M by the dropwise addition of a stock solution (room temperature) of 3.9 M ultrapure ammonium sulfate. The medium was stirred for an additional 5 minutes at 4°C, filtered as before, and applied to a Butyl Sepharose<sup>®</sup> 4 Fast Flow column (81 ml column volume, 2.5 x 16.5 cm; Pharmacia, Uppsala, Sweden) that had been equilibrated in 10 mM MES-Tris, pH 5.6, containing 0.66 M ammonium sulfate (buffer A). The chromatography was performed at 4°C on a Gradi-Frac™

- 27 -

System (Pharmacia, Uppsala, Sweden) equipped with in-line UV (280 nm) and conductivity monitors for assessing total protein and salt concentration, respectively. After sample application at a flow rate of 10 ml/min, the column was washed with 10 column volumes of buffer A. The  $\alpha$ -gal A was eluted from the Butyl Sepharose® column with a 14 column volume linear gradient from buffer A (containing ammonium sulfate) to 10 mM MES-Tris, pH 5.6 (no ammonium sulfate). Fractions were assayed for  $\alpha$ -gal A activity by the 4-MUF-gal assay, and those containing appreciable enzyme activity were pooled. As seen in Fig. 6 and the purification summary (Table 3), this step removes approximately 99% of the contaminating protein (pre-column sample=8.14 g total protein; post-column sample=0.0638 g total protein).

- 28 -

Table 4: Purification of  $\alpha$ -Gal A from the Conditioned Medium of Stably Transfected Human Fibroblasts

Purification Step	Volume (ml)	$\alpha$ -Gal A Activity ( $\times 10^6$ Units)	Total Protein (mg)	Specific Activity ( $\times 10^6$ Units /mg)	Fold Purification (Cumulative)	Percent Recovery
5 Culture supernatant	1340	14.6	8140	0.0018	=1	=100
10 Butyl Sepharose	417	14.1	63.8	0.221	123	96.6
Heparin Sepharose	134	12.1	14.6	0.829	436	82.9
15 Hydroxyapatite	47	9.73	4.46	2.18	1220	66.6
Q Sepharose	31.5	8.91	3.31	2.69	1503	61.0
20 Superdex 200	10	8.58	2.93	2.92	1634	59.0

B. Use of Heparin Sepharose® Chromatography as a Step for Purification of  $\alpha$ -Gal A

The Butyl Sepharose® column peak fractions were dialyzed at 4°C against (4 liters) of 10 mM MES-Tris, pH 5.6 (changed once). The conductivity of the dialysate was adjusted to 1.0 mMH $\Omega$  at 4°C by addition of H $_2$ O or NaCl as necessary. Afterward, the sample was applied to a column of Heparin Sepharose® 6 Fast Flow (Pharmacia, Uppsala, Sweden; 29 ml column volume, 2.5 x 6 cm) that had been equilibrated in 10 mM MES-Tris, pH 5.6, containing 9 mM NaCl (buffer B). This was done at 4°C at a flow rate of 10 ml/min. In-line UV (280 nm) and

- 29 -

conductivity monitors measured total protein and salt concentration. After the sample was applied, the column was washed with 10 column volumes of buffer B followed by a 3 column volume linear gradient to 8% buffer C/92%  
5 buffer B (where buffer C is 10 mM MES-Tris, pH 5.6, containing 250 mM NaCl) and a 10 column volume wash with 8% buffer C. This was followed by elution of  $\alpha$ -gal A with a 1.5 column volume linear gradient to 29% buffer C and a subsequent 10 column volume linear gradient to  
10 35% buffer C. Fractions were assayed for  $\alpha$ -gal A activity, and those containing appreciable activity were pooled.

C. Use of Hydroxyapatite

Chromatography as a Step for

15 Purification of  $\alpha$ -Gal A

The heparin pool was filtered and applied directly to a column of Ceramic Hydroxyapatite HC (40  $\mu$ m; American International Chemical, Natick, MA; 12 ml column volume, 1.5 x 6.8 cm) that had been equilibrated in 1 mM  
20 sodium phosphate, pH 6.0 (buffer D). The chromatography was performed at room temperature on a hybrid Gradi-Frac<sup>™</sup>/FPLC<sup>®</sup> System (Pharmacia, Uppsala, Sweden) equipped with in-line UV (280 nm) and conductivity monitors. After the sample was applied (5 ml/min), the column was  
25 washed with 10 column volumes of buffer D. The  $\alpha$ -gal A was eluted with a 7 column volume linear gradient to 42% buffer E/58% buffer D (where buffer E is 250 mM sodium phosphate, pH 6.0) followed by a 10 column volume gradient to 52% buffer E. Fractions were assayed for  $\alpha$ -  
30 gal A activity, and the fractions containing appreciable activity were pooled.



- 30 -

D. Use of Q Sepharose® Anion  
Exchange Chromatography as a Step  
for Purification of  $\alpha$ -Gal A

The hydroxyapatite pool was diluted  
5 approximately 1.5 fold with H<sub>2</sub>O to a final conductivity  
of 3.4-3.6 mMHO at room temperature. After filtering,  
the sample was applied to a column of Q Sepharose® HP  
(Pharmacia, Uppsala, Sweden; 5.1 ml column volume,  
1.5 x 2.9 cm) equilibrated in 10% buffer G/90% buffer F,  
10 where buffer F is 25 M sodium phosphate, pH 6.0, and  
buffer G is 25 mM sodium phosphate, pH 6.0, 250 mM NaCl.  
The chromatography was performed at room temperature on  
the Gradi-Frac™/FPLC® hybrid system (Pharmacia, Uppsala,  
Sweden), and total protein and salt concentrations were  
15 monitored by the in-line monitors. The sample was  
applied at a flow rate of 5 ml/min, then the following  
steps were performed: (1) a 5 column volume wash at  
10% buffer G, (2) a 7 column volume wash at 12% buffer G,  
(3) a 3 column volume linear gradient to 50% buffer G,  
20 (4) a 10 column volume linear gradient to 53% buffer G,  
(5) a 3 column volume gradient to 100% buffer G, and  
(6) a 10 column volume wash at 100% buffer G. The  $\alpha$ -  
gal A eluted primarily during steps 3 and 4. Fractions  
containing appreciable activity were pooled (the  
25 "Q pool").

E. Use of Superdex®-200 Gel  
Filtration Chromatography as a Step  
for Purification of  $\alpha$ -Gal A

The Q pool was concentrated approximately 5-  
30 fold using Centriprep®-10 centrifugal concentrator units  
(Amicon, Beverly, MA), and applied to a column of  
Superdex® 200 (Pharmacia, Uppsala, Sweden; 189 ml column  
volume, 1.6 x 94 cm). The column was equilibrated and  
eluted with 25 mM sodium phosphate, pH 6.0, containing  
35 150 mM NaCl. The chromatography was performed on an

- 31 -

FPLC® system (Pharmacia, Uppsala, Sweden) at room temperature using an in-line UV monitor (280 nm) to follow elution of the protein. The volume of the sample applied to the column was  $\leq 2$  ml, the flow rate was 0.5 ml/min, and the fraction size was 2 ml. Multiple column runs were performed; fractions were assayed for  $\alpha$ -gal A activity and fractions containing appreciable activity were pooled.

The pooled fractions from the Superdex® 200 column were concentrated using Centriprep-10 units, aliquoted, snap-frozen, and stored at  $-80^{\circ}\text{C}$  for short periods of time. A summary of this example of  $\alpha$ -gal A purification is shown in Table 3. The final yield of  $\alpha$ -gal A was 59% of the starting material activity, and the specific activity of the purified product was  $2.92 \times 10^6$  units/mg protein. The resulting product showed a high level of purity after electrophoresis under reducing conditions on a 4-15% SDS-polyacrylamide gel, which was subsequently silver-stained.

### 20 Example III. Formulation and Storage of Purified $\alpha$ -Gal A

Highly purified  $\alpha$ -gal A is not stable for extended periods of time when stored as a dilute solution of purified protein ( $\leq 1$  mg protein/ml). Therefore, a formulation was devised to improve stability during prolonged storage, i.e. storage lasting several weeks to at least several months. The purified enzyme was concentrated to at least 1 mg/ml using a centrifugal concentrator (in enzyme buffer consisting of 25 mM sodium phosphate (pH 6.0) and 150 mM NaCl). Human serum albumin (HSA; Buminat®, Baxter-Hyland) was added to a final concentration of 2.5 mg/ml. The protein solution was then sterile filtered using a  $0.2 \mu\text{m}$  cellulose acetate filter (Schleicher and Schuell) attached to a syringe. The  $\alpha$ -gal A solution was dispensed into sterile, pyrogen-

- 32 -

free glass vials, sealed with a Teflon cap, snap-frozen, and stored at  $-20^{\circ}\text{C}$ .

Stability of the  $\alpha$ -gal A activity was evaluated over a three month period using the 4-MUF-gal assay. The data presented in Table 5 demonstrate that there was no loss of enzyme activity over the test period. The acidic pH of the formulation ( $< 6.5$ ) is critical to the stability of the highly purified enzyme.

Table 5: Stability of Formulated  $\alpha$ -Gal A at  $-20^{\circ}\text{C}$

10	Sample	Specific Activity (Units/mg total protein)
	time 0	$2.24 \times 10^6 \pm 0.33 \times 10^6$
	week 1	$2.40 \times 10^6 \pm 0.25 \times 10^6$
	week 2	$2.42 \times 10^6 \pm 0.21 \times 10^6$
	week 3	$2.37 \times 10^6 \pm 0.05 \times 10^6$
15	month 1	$2.39 \times 10^6 \pm 0.16 \times 10^6$
	month 2	$2.31 \times 10^6 \pm 0.26 \times 10^6$
	month 3	$2.29 \times 10^6 \pm 0.17 \times 10^6$

- 33 -

Example IV.  $\alpha$ -Gal A Produced by Human Cell Strains is Suitable for Treatment of  $\alpha$ -Gal A Deficiency

The structural and functional properties of purified human  $\alpha$ -gal A prepared in accordance with the invention were investigated in order to demonstrate that the DNA molecules described herein and the corresponding expressed glycoproteins produced by transfected human cell strains can be used in gene or enzyme replacement therapies, respectively.

10           A. Size of  $\alpha$ -Gal A Produced by Stably Transfected Human Cells in Culture

The molecular mass of  $\alpha$ -gal A was estimated by MALDI-TOF mass spectrometry. These results demonstrate that the molecular mass of the dimer is 102,353 Da, while 15 that of the monomer is 51,002 Da. The expected molecular mass of the monomer, based on amino acid composition, is 45,400 Da. Therefore, it can be inferred that the carbohydrate content of the enzyme accounts for up to 5,600 Da of the molecular weight.

20           The results of standard amino acid analysis performed on the purified protein are consistent with the conclusion that the protein produced by transfected human cells is identical to the protein purified from human tissues at the amino acid level.

25           B. N-Terminal Processing of  $\alpha$ -Gal A Produced by Stably Transfected Human Cells

The human  $\alpha$ -gal A cDNA nucleotide sequence encodes 429 amino acids. The 31 N-terminal amino acids constitute a signal peptide sequence, which is cleaved as 30 the nascent protein transits the endoplasmic reticulum membrane (LeDonne et al., Arch. Biochem. Biophys. 224:186, 1983; Lemansky et al., J. Biol. Chem. 262:2062, 1987). In order to confirm that  $\alpha$ -gal A is properly processed when associated with an heterologous signal 35 peptide sequence (for example, the human growth hormone

- 34 -

signal sequence) and expressed in transfected human fibroblasts, ten N-terminal amino acids of the secreted protein were microsequenced. Samples were electrophoresed by SDS-PAGE and transferred to ProBlott® (ABI, Foster City, CA) using a 10 mM CAPS (pH 11.0), 10% methanol buffer system. The protein on the ProBlott® was visualized by Coomassie staining, and an appropriately sized (50 kDa) band was excised. N-terminal sequence was obtained using an Applied Biosystems pulse-liquid phase amino acid sequenator that performs automated Edman degradation. The N-terminal sequence obtained, LDNGLARTPT (SEQ ID NO:28), is consistent with proper cleavage of the signal peptide and matches the N-terminal sequence predicted for the secreted protein.

C. C-Terminal Amino Acid of  $\alpha$ -Gal A  
Produced by Stably Transfected Human Cells

The C-terminal amino acid residue of secreted  $\alpha$ -gal A produced in accordance with the invention was identified using an automated Hewlett Packard C-terminal sequencer. The results indicated a leucine residue at the C-terminus, which agrees with the C-terminal amino acid predicted by the DNA sequence.

D. Carbohydrate Modification of  $\alpha$ -Gal A  
Produced by Stably Transfected Human Cells

The glycosylation pattern of  $\alpha$ -gal A produced in accordance with the invention was also evaluated. Proper glycosylation is important for optimal *in vivo* activity of  $\alpha$ -gal A;  $\alpha$ -gal A expressed in non-glycosylating systems is inactive or unstable (Hantzopolous et al., Gene 57:159, 1987). Glycosylation is also important for the internalization of  $\alpha$ -gal A into the desired target cells, and affects the circulating half-life of the enzyme *in vivo*. On each subunit of  $\alpha$ -gal A there are four sites available for addition of

- 35 -

asparagine-linked carbohydrate chains, of which only three are occupied (Desnick et al., In The Metabolic and Molecular Bases of Inherited Disease, pp 2741-2780, McGraw Hill, New York, 1995).

5           A sample of  $\alpha$ -gal A produced by stably transfected cells was treated with neuraminidase, which is isolated from *A. urafaciens*, (Boehringer-Mannheim, Indianapolis, IN) to remove sialic acid. This reaction was performed by treating 5  $\mu$ g of  $\alpha$ -gal A overnight with  
10 10 mU of neuraminidase at room temperature in a total volume of 10  $\mu$ l of acetate buffered saline (ABS, 20 mM sodium acetate, pH. 5.2, 150 mM NaCl).

Purified  $\alpha$ -gal A produced by stably transfected cells was also dephosphorylated using alkaline  
15 phosphatase (calf intestinal alkaline phosphatase, Boehringer-Mannheim, Indianapolis, IN), by treating 5  $\mu$ g of  $\alpha$ -gal A overnight at room temperature with 15 U of alkaline phosphatase in ABS (pH raised to 7.5 with 1 M Tris).

20           The samples were analyzed by Western blot with an  $\alpha$ -gal A-specific antibody. The antibody used was a rabbit polyclonal anti-peptide antibody, which was produced using a peptide representing amino acids 68-81 of  $\alpha$ -gal A as an immunogen. Following transfer of the  
25 protein to PVDF (Millipore, Bedford, MA), the membrane was probed with a 1:2000 dilution of the anti-serum in 2.5% blotto (non-fat dry milk in 20 mM Tris-HCl, pH 7.5, 0.05% Tween-20). This was followed by detection with goat anti-rabbit IgG conjugated to horseradish peroxidase  
30 (Organon Teknika/Cappel, Durham, NC; 1:5000 dilution) and reagents of the ECL chemiluminescence kit (Amersham, Arlington Heights, IN).

Treatment of  $\alpha$ -gal A with neuraminidase results in a slight shift in molecular mass (approximately 1500-  
35 2000 Da or 4-6 sialic acids/monomer), suggesting that

- 36 -

there is extensive modification of  $\alpha$ -gal A with sialic acid. For reference, the plasma form of  $\alpha$ -gal A has 5-6 sialic acid residues per monomer, and the placental form has 0.5-1.0 sialic acid residues per monomer (Bishop  
5 et al., J. Biol. Chem. 256:1307, 1981).

Another method used to examine the sialic acid and mannose-6-phosphate modifications of  $\alpha$ -gal A was isoelectric focusing (IEF), where the samples are separated on the basis of their isoelectric point (pI) or  
10 net charge. Thus, removal of charged residues such as sialic acid or phosphate from  $\alpha$ -gal A would be expected to alter the mobility of the protein in the IEF system.

To perform the IEF experiment, samples of  $\alpha$ -gal A produced in accordance with the invention were  
15 treated with neuraminidase and alkaline phosphatase, mixed 1:1 with 2X Novex sample buffer (with 8 M urea, pH 3.0-7.0), and loaded onto a 6 M urea IEF gel (5.5% polyacrylamide) made using Pharmalyte® (Pharmacia, Uppsala, Sweden; pH 3.0-6.5; Pharmalyte® 4-6.5 and 2.5-  
20 5.5, 0.25 ml each per gel). Isoelectric point standards (Bio-Rad) were also included. Following electrophoresis, the gel was transferred to PVDF, and Western blot analysis performed as described above.

The  $\alpha$ -gal A produced by stably transfected  
25 human fibroblasts consisted of three major isoforms with a pI range of approximately 4.4-4.65. These values are similar to the pIs of the plasma and splenic forms of  $\alpha$ -gal A (Bishop et al., J. Biol. Chem. 256:1307, 1981). Neuraminidase treatment of the enzyme increased the pI of  
30 all three isoforms, indicating that all were modified to some extent by sialic acid. These data suggest that the  $\alpha$ -gal A produced by stably transfected human cells should have a desirable plasma half-life, indicating that this material is well suited for pharmacologic use. Further,  
35 treatment of neuraminidase-treated  $\alpha$ -gal A with alkaline

- 37 -

phosphatase further increased the pI of a portion of the protein to approximately 5.0-5.1, indicating that the enzyme bears one or more mannose-6-phosphate residues. This modification is significant in that it is required for efficient internalization of  $\alpha$ -gal A by the target cells.

#### E. Specific Activity of $\alpha$ -gal A

##### Purified from Stably Transfected Fibroblasts

The potency or specific activity of purified  $\alpha$ -gal A is calculated by measuring both the catalytic activity of the enzyme (with the 4-MUF-gal assay), and the protein concentration. The protein concentration can be determined by any standard method, such as with the BCA system (Pierce), or by measuring the absorbance at 280 nm and using the mg/ml extinction coefficient of 2.3 (determined from amino acid analysis) to calculate the value. Using these techniques, the specific activity of  $\alpha$ -gal A purified from the conditioned medium of transfected human fibroblasts is  $2.2-2.9 \times 10^6$  units/mg of protein, which is comparable to the specific activity of  $\alpha$ -gal A that is purified from human tissues (Bishop et al., J. Biol. Chem. 256:1301, 1981).

#### F. Mannose or Mannose-6-Phosphate Mediated Internalization of $\alpha$ -Gal A

In order for the  $\alpha$ -gal A produced by stably transfected cells to be an effective therapeutic agent for  $\alpha$ -gal A deficiencies, the enzyme must be internalized by the affected cells.  $\alpha$ -Gal A is not active at physiological pH levels, and is unlikely to be effective in the blood or interstitial fluids. It metabolizes accumulated lipid substrates optimally only when internalized in the acidic environment of the lysosome. This internalization is mediated by the binding of  $\alpha$ -gal A to mannose-6-phosphate (M6P) receptors, which are expressed on the cell surface and deliver the enzyme to



- 38 -

the lysosome via the endocytic pathway. The M6P receptor is ubiquitously expressed; most somatic cells express it to some extent. The mannose receptor, which is specific for exposed mannose residues on glycoproteins, is less prevalent. The latter receptors are generally found only on macrophage and macrophage-like cells, and provide an additional means of  $\alpha$ -gal A entry into these cell types.

In order to demonstrate M6P-mediated internalization of  $\alpha$ -gal A, skin fibroblasts from a Fabry disease patient (NIGMS Human Genetic Mutant Cell Repository) were cultured overnight in the presence of increasing concentrations of purified  $\alpha$ -gal A of the invention. Some of the samples contained 5 mM soluble M6P, which competitively inhibits binding to, and as a result, internalization by, the mannose-6 phosphate receptor. Other samples contained 30  $\mu$ g/ml mannan, which inhibits binding to, and as a result, internalization by, the mannose receptor. Following incubation, the cells were washed and harvested by scraping into lysis buffer (10 mM Tris, pH 7.2, 100 mM NaCl, 5 mM EDTA, 2 mM Pefabloc™ (Boehringer-Mannheim, Indianapolis, IN) and 1% NP-40). The lysed samples were then assayed for protein concentration and  $\alpha$ -gal A activity. The results are expressed as units of  $\alpha$ -gal A activity/mg cell protein. The Fabry cells internalized  $\alpha$ -gal A in a dose-dependent manner (Fig. 7). This internalization was inhibited by mannose-6 phosphate, but there was no inhibition with mannan. Therefore, internalization of  $\alpha$ -gal A in Fabry fibroblasts is mediated by the mannose-6 phosphate receptor, but not by the mannose receptor.

$\alpha$ -gal A is also internalized *in vitro* by endothelial cells, important target cells for the treatment of Fabry disease. Human umbilical vein endothelial cells (HUVECs) were cultured overnight with 7500 units of  $\alpha$ -gal A; some of the wells contained M6P.

- 39 -

After the incubation period, cells were harvested and assayed for  $\alpha$ -gal A as described above. The cells incubated with  $\alpha$ -gal A only had enzyme levels almost 10-fold above those of control (no incubation with  $\alpha$ -gal A) cells. M6P inhibited the intracellular accumulation of  $\alpha$ -gal A, suggesting that the internalization of  $\alpha$ -gal A by HUVECs is mediated by the M6P receptor. Thus, the human  $\alpha$ -gal A of the invention is internalized by clinically relevant cells.

Few cultured human cell lines are known to express the mannose receptor. However, a mouse macrophage-like cell line (J774.E) which bears mannose receptors but few if any mannose 6-phosphate receptors can be used to determine whether purified  $\alpha$ -gal A of the invention is internalized via the mannose receptor (Diment et al., J. Leukocyte Biol. 42:485-490, 1987). J774.E cells were cultured overnight in the presence of 10,000 units/ml  $\alpha$ -gal A. Selected samples also contained 2 mM M6P, and others contained 100  $\mu$ g/ml mannan. The cells were washed and harvested as described above, and the total protein and  $\alpha$ -gal A activity of each sample was determined. The results are shown in Table 5. M6P does not inhibit the uptake of  $\alpha$ -gal A by these cells, while mannan decreases the accumulated  $\alpha$ -gal A levels by 75%. Thus, the  $\alpha$ -gal A of the invention may be internalized by the mannose receptor in cell types that express this particular cell surface receptor.

- 40 -

Table 6. Internalization of  $\alpha$ -Gal A by J774.E Cells.

$\alpha$ -Gal A Activity (units/mg total protein)				
	No additions	+ $\alpha$ -gal A	+ $\alpha$ -gal A, + M6P	+ $\alpha$ -gal A, + mannan
J774.E	409 $\pm$ 25	6444 $\pm$ 554	6297 $\pm$ 674	1654 $\pm$ 323

[mannan] = 100  $\mu$ g/ml5 [M6P] = 2 mM or 660  $\mu$ g/ml

These experiments demonstrate that the  $\alpha$ -gal A produced by stably transfected human cells may be internalized by cells via the mannose or mannose-6-phosphate receptor.

10 G. Correction of Fabry Fibroblasts by Human Fibroblasts Expressing  $\alpha$ -Gal A

For gene therapy, an implant of autologous cells producing  $\alpha$ -gal A must produce the enzyme in a form modified appropriately to "correct" the  $\alpha$ -gal A

15 deficiency in target cells. To assess the effect of  $\alpha$ -gal A production by transfected human fibroblasts on Fabry cells, fibroblasts harvested from Fabry disease patients (NIGMS Human Genetics Mutant Cell Repository) were co-cultured with an  $\alpha$ -gal A-producing cell strain

20 (BRS-11) in Transwells<sup>®</sup> (Costar, Cambridge, MA). The experimental scheme is depicted in Fig. 8. Fabry cells were cultured in 12-well tissue culture dishes, some of which contained inserts (Transwells<sup>®</sup>, 0.4  $\mu$ m pore size) having a surface on which cells can be grown. The growth

25 matrix of the insert is porous and allows macromolecules to pass from the upper to the lower milieu. One set of inserts contained normal human foreskin (HF) fibroblasts,

- 41 -

which secrete minimal levels of  $\alpha$ -gal A, while another set contained the stably transfected human fibroblast strain, BRS-11, which secretes large amounts of  $\alpha$ -gal A. In the wells co-cultured with  $\alpha$ -gal A-producing cells,  $\alpha$ -gal A can enter the medium bathing the Fabry cells, and potentially be internalized by the Fabry cells.

The data in Table 7 show that Fabry cells internalized the secreted  $\alpha$ -gal A. The intracellular levels of  $\alpha$ -gal A were monitored for three days. Those cells cultured alone (no insert) or in the presence of non-transfected foreskin fibroblasts (HF insert) had very low intracellular levels of  $\alpha$ -gal A activity. The Fabry cells cultured with the  $\alpha$ -gal A-producing (BRS-11 insert) cells, however, exhibited enzyme levels similar to those of normal cells by the end of Day 2 (normal fibroblasts have 25-80 units  $\alpha$ -gal A/mg protein). That the correction is attributable to  $\alpha$ -gal A taken up via the M6P receptor is demonstrated by its inhibition with mannose-6-phosphate (BRS-11 insert + M6P).

Table 7. Correction of Fabry Fibroblasts by Human Fibroblasts Expressing  $\alpha$ -Gal A

$\alpha$ -Gal A Activity (units/mg total protein)

Time	no insert	HF insert	BRS-11 insert	BRS-11 insert+M6P
Day 1	2 $\pm$ 1	2 $\pm$ 1	13 $\pm$ 1	4 $\pm$ 1
Day 2	2 $\pm$ 1	2 $\pm$ 1	40 $\pm$ 11	6 $\pm$ 2
Day 3	2 $\pm$ 1	5 $\pm$ 1	85 $\pm$ 1	9 $\pm$ 1

#### H. Utility of Other Cell Types

Other cell types can be used in the method described herein. The cells can be obtained from a variety of tissues and include all cell types that can be

- 42 -

maintained in culture. For example, primary and secondary cells that can be transfected by the present method include human fibroblasts, keratinocytes, epithelial cells (e.g., mammary or intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes and bone marrow cells), muscle cells, and precursors of these somatic cell types. Fibroblasts are of particular interest. Primary cells are preferably obtained from the individual to whom the transfected primary or secondary cells are to be administered, so that they will not be rejected by the patient's immune system. However, if proper attention is paid to avoiding or suppressing immunorejection (as described below), cells of a human donor other than the patient can be used as well. This would permit use of cells from an standardized, established, stably transfected cell line in all patients.

#### I. Administration of $\alpha$ -Gal A-Expressing Cells

The cells described above may be introduced into an individual, through various standardized routes of administration, so that they will reside in, for example, the renal subcapsule, a subcutaneous compartment, the central nervous system, the intrathecal space, the liver, the intraperitoneal cavity, or within a muscle. The cells may also be injected intravenously or intra-arterially so that they circulate within the individual's bloodstream. Once implanted in the individual, the transfected cells will produce and secrete the therapeutic product, glycosylated human  $\alpha$ -gal A.

The number of genetically modified cells that will be introduced into the individual will vary, but can be determined by skilled artisans. The age, weight, sex, and general physical condition of each patient, as well

- 43 -

as the volume of distribution, the half-life and bioavailability of the enzyme, and the *in vivo* productivity of the genetically modified cells, will be among the primary considerations in determining dosage and route of administration. Typically, between one million and one billion cells will be used, with expression levels ranging from 100-100,000 units per  $10^6$  cells per day. If necessary, the procedure may be repeated or modified until the desired result, for example, relief from the symptoms associated with Fabry disease, is achieved.

As described above, the cells used will generally be patient-specific, i.e., obtained from the individual to whom the transfected primary or secondary cells are to be administered, so that they will not be rejected by the patient's immune system. If, however, this scenario is not possible or desirable, cells may be obtained from another individual, genetically modified as described herein, and implanted into the patient who is suffering from  $\alpha$ -gal A deficiency.

The use of cells from an individual other than the recipient might require administration of an immunosuppressant, alteration of histocompatibility antigens, or use of a barrier device to prevent rejection of the implanted cells. The barrier device will be made of a material (e.g., a membrane such as XM-50 from Amicon, Beverly, MA) that permits the secreted product to pass into the recipient's circulation or tissues, but prevents contact between the implanted cells and the recipient's immune system, and thus prevents an immune response to (and possible rejection of) the cells by the recipient. For further guidance regarding gene therapy, see Selden et al. (WO 93/09222).

The cells may alternatively be embedded in a matrix or gel material, such as described in co-owned

- 44 -

U.S.S.N. 08/548/002, which describes the use of hybrid matrix implants, or in Jain et al. (PCT application WO 95/19430), which describes macroencapsulation of secretory cells in a hydrophilic gel material (each of which is hereby incorporated by reference).

J. Pharmaceutical Formulation for Conventional Administration of  $\alpha$ -Gal A Protein

The  $\alpha$ -gal A protein that is expressed and secreted by stably transfected (or otherwise genetically modified) human cells and purified as described herein may be administered to individuals who produce insufficient or defective  $\alpha$ -gal A protein. The protein may be administered in a pharmaceutically acceptable carrier, at a pH below 6.5, e.g. in a formulation as described in Example III. Examples of excipients which may be included with the formulation are buffers such as citrate buffer, phosphate buffer, acetate buffer, and bicarbonate buffer, amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins, such as serum albumin and gelatin, EDTA, sodium chloride, liposomes, polyvinylpyrrolidone, mannitol, sorbitol, glycerol, propylene glycol and polyethylene glycol (e.g., PEG-4000, PEG-6000). The route of administration may be, for example, intravenous, intra-arterial, subcutaneous, intraperitoneal, intracerebral, intramuscular, intrapulmonary, or transmucosal. The route of administration and the amount of protein delivered will be determined by factors that are well within the ability of skilled artisans to assess. Furthermore, skilled artisans are aware that the route of administration and dosage of a therapeutic protein may be varied for a given patient until a therapeutic dosage level is obtained. Typically, doses of  $\alpha$ -gal A of 0.01-100 mg/kg of body weight will be administered. It is expected that

- 45 -

regularly repeated doses of the protein will be necessary over the life of the patient.

K. Treatment of Other Conditions  
Caused by Enzyme Deficiencies

5 It is likely that other conditions caused by deficiencies in lysosomal storage enzymes other than  $\alpha$ -gal A will be amenable to treatment by methods comparable to those described herein. In these cases, DNA which encodes a functional form of the deficient  
 10 enzyme would be substituted for the DNA encoding  $\alpha$ -gal A in the expression constructs disclosed herein. Examples of enzyme deficiency syndromes that have been identified, and that may be amenable to treatment as described herein, are shown in Table 8. The information in this  
 15 Table is taken from E. Neufeld (Ann. Rev. Biochem. 60:257-280, 1991), which is hereby incorporated by reference.

Table 8. Summary of Lysosomal Storage Disorders

---

20 Disorder	Primary deficiency [secondary deficiency]
<u>Disorders of sphingolipid degradation</u>	
Fabry disease	$\alpha$ -galactosidase
Farber disease	ceramidase
25 Gaucher disease	glucocerebrosidase
G <sub>M1</sub> gangliosidosis	$\beta$ -galactosidase



- 46 -

G <sub>M2</sub> gangliosidoses subunit	$\beta$ -hexosaminidase, $\alpha$ -
Tay-Sachs disease	[hexosaminidase A]
Sandhoff disease	$\beta$ -hexosaminidase, $\beta$ -
5 subunit	[hexosaminidases A and B]
Activator deficiency Krabbe disease	G <sub>M2</sub> activator galactosylceramidase
10 Metachromatic leukodystrophy enzyme-deficient form activator-deficient form activator/saposin	arylsulfatase A sulfatide
Mucopolipidosis IV	primary defect unknown [ganglioside sialidase]
15 Multiple sulfatase deficiency sulfatases]	primary defect unknown [deficiency of all
Niemann-Pick disease	sphingomyelinase
20 Schindler disease acetylgalactosaminidase	$\alpha$ -N-
<u>Disorders of glycoprotein degradation</u>	
Aspartylglycosaminuria	aspartylglycosaminidase
Fucosidosis	$\alpha$ -L-fucosidase

- 47 -

Galactosialidosis	protective
protein/cathepsin	
sialidase]	[ $\beta$ -galactosidase and
5 $\alpha$ -Mannosidosis	$\alpha$ -mannosidase
$\beta$ -Mannosidosis	$\beta$ -mannosidase
Sialidosis	sialidase
<u>Disorders of glycosaminoglycan degradation</u>	
Hunter syndrome	iduronate sulfatase
10 Hurler and Scheie syndromes	$\alpha$ -L-iduronidase
Maroteaux-Lamy syndrome	GalNAc 4-
sulfatase/arylsulfatase	
Morquio syndrome	
A-subtype	Gal 6-sulfatase
15 B-subtype	$\beta$ -galactosidase
Sanfilippo syndrome	
A-subtype	heparan N-sulfatase
B-subtype	$\alpha$ -N-acetylglucosaminidase
C-subtype	AcetylCoA: glucosamine
20	N-acetyltransferase

- 48 -

D-subtype	GlcNAc 6-sulfatase
Sly syndrome	$\beta$ -glucuronidase
<u>Other single enzyme deficiency disorders</u>	

Pompe disease (glycogenosis II)	$\alpha$ -glucosidase
5 Wolman disease	acid lipase

## Disorders of lysosomal enzyme biosynthesis

	I-cell disease and pseudoHurler	6-phospho- <i>N</i> -
	acetylglucosamine	
		transferase
10	polydystrophy	[mislocalization of many
	lysosomal	
		enzymes]

## Disorders of lysosomal membrane transport

Cystinosis	cystine transport
15 Sialic storage and Salla diseases	sialic acid transport

- 49 -

### V. Other Embodiments

The invention described herein has been exemplified in part by methods of treatment that employ cells which express a particular gene product following  
5 transfection, i.e., after introduction of a construct encoding the gene product and having regulatory elements that control expression of the coding sequence. These methods may also be carried out using cells that have been genetically modified by other procedures, including  
10 gene targeting and gene activation (see Treco et al. (WO 95/31560, herein incorporated by reference; see also Selden et al. WO 93/09222).

The hGH signal peptide can be used with heterologous proteins other than  $\alpha$ -gal A, to increase the  
15 level of expression and secretion of the heterologous protein. Examples of such proteins include  $\alpha$ -1 antitrypsin, antithrombin III, apolipoprotein E, apolipoprotein A-1, blood clotting factors V, VII, VIII, IX, X, and XIII, bone growth factor-2, bone growth  
20 factor-7, calcitonin, catalytic antibodies, DNase, erythropoietin, FSH- $\beta$ , globins, glucagon, glucocerebrosidase, G-CSF, GM-CSF, growth hormone, immune response modifiers, immunoglobulins, insulin, insulinotropin, insulin-like growth factors,  
25 interferon- $\beta$ , interferon- $\beta$  nerve growth factors, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-11, interleukin-12, IL-2 receptor, IL-1 receptor antagonists, low density lipoprotein receptor, M-CSF, parathyroid hormone, protein  
30 kinase C, soluble CD4, superoxide dismutase, tissue plasminogen activator, TGF- $\beta$ , tumor necrosis factor, TSH $\beta$ , tyrosine hydroxylase, and urokinase.

Other embodiments are within the following claims.

- 50 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: Transkaryotic Therapies, Inc.
- (ii) TITLE OF THE INVENTION: THERAPY FOR  $\alpha$ -GALACTOSIDASE A DEFICIENCY
- (iii) NUMBER OF SEQUENCES: 28
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Fish & Richardson P.C.
  - (B) STREET: 225 Franklin Street
  - (C) CITY: Boston
  - (D) STATE: MA
  - (E) COUNTRY: US
  - (F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: Windows95
  - (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: PCT/US97/16603
  - (B) FILING DATE: 12-SEP-1997
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/712,614
  - (B) FILING DATE: 13-SEP-1996
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Fraser, Janis K.
  - (B) REGISTRATION NUMBER: 34,819
  - (C) REFERENCE/DOCKET NUMBER: 07236/003WO1
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 617/542-5070
  - (B) TELEFAX: 617/542-8906
  - (C) TELEX: 200154

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGGGCTGTA GCTATGATAA AC

22

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single

- 51 -

- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
- TCTAGCTGAA GCAAAACAGT G 21
- (2) INFORMATION FOR SEQ ID NO:3:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
- ATTGGTCCGC CCCTGAGGT 19
- (2) INFORMATION FOR SEQ ID NO:4:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
- TGATGCAGGA ATCTGGCTCT 20
- (2) INFORMATION FOR SEQ ID NO:5:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
- TTTTGGATCC ACCATGGCTA 20
- (2) INFORMATION FOR SEQ ID NO:6:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
- TTTTGCCGGC ACTGCCCTCT TGAA 24
- (2) INFORMATION FOR SEQ ID NO:7:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- 52 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTTTCAGCTG GACAATGGAT TGGC

24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTTTGCTAGC TGGCGAATCC

20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTTTGGATCC GTGTCCCATG GTGTTTCCAA

30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTTGGATCC GCAGTCGTGG CCACTACC

28

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTAGTCCTAG GA

12

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- 53 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTTTGAGCAC AGAGCCTCGC CT

22

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTTTGGATCC GGTGAGCTGC GAGAATAGCC

30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 76 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGGCCCCCAG CCCCAGCCCT CCCATTGGTG GAGGCCCTTT TGGAGGCACC CTAGGGCCAG  
GAAACTTTTG CCGTAT60  
76

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAATAGGGCA GATCCGGGCT TTATTATTTT AGCACCACGG CCGCGAGAC CGCGTCCGCC  
CCGCGAGCA60  
69

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 86 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGCCCTATTT ATACGGCAAA AGTTTCCTGG CCCTAGGGTG CCTCCAAAAG GGCCTCCACC  
AATGGGAGGG CTGGGGCTGG GGGCCC60  
86

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single



- 54 -

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGCGGGGCGG ACGCGGTCTC GGCGGCCGTG GTGCTAAAT AATAAGCCCC GGATC 55

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1343 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCGCGGGAAA	TTTATGCTGT	CCGGTCACCG	TGACAATGCA	GCTGAGGAAC	CCAGAACTAC	60
ATCTGGGCTG	CGCGCTTGCG	CTTCGCTTCC	TGGCCCTCGT	TTCCTGGGAC	ATCCCTGGGG	120
CTAGAGCACT	GGACAATGGA	TTGGCAAGGA	CGCCTACCAT	GGGCTGGCTG	CACTGGGAGC	180
GCTTCATGTG	CAACCTTGAC	TGCCAGGAAG	AGCCAGATTG	CTGCATCAGT	GAGAAGCTCT	240
TCATGGAGAT	GGCAGAGCTC	ATGGTCTCAG	AAGGCTGGAA	GGATGCAGGT	TATGAGTACC	300
TCTGCATTGA	TGACTGTTGG	ATGGCTCCCC	AAAGAGATTG	AGAAGGCAGA	CTTCAGGCAG	360
ACCCTCAGCG	CTTTCCTCAT	GGGATTCGCC	AGCTAGCTAA	TTATGTTTAC	AGCAAAGGAC	420
TGAAGCTAGG	GATTTATGCA	GATGTTGGAA	ATAAAACCTG	CGCAGGCTTC	CCTGGGAGTT	480
TTGGATACTA	CGACATTGAT	GCCCAGACCT	TTGCTGACTG	GGGAGTAGAT	CTGCTAAAT	540
TTGATGGTTG	TTACTGTGAC	AGTTTGGAAA	ATTTGGCAGA	TGTTTATAAG	CACATGTCCT	600
TGGCCCTGAA	TAGGACTGGC	AGAAGCATTG	TGTACTCCTG	TGAGTGGCCT	CTTTATATGT	660
GGCCCTTTCA	AAAGCCCAAT	TATACAGAAA	TCCGACAGTA	CTGCAATCAC	TGGCGAAATT	720
TTGCTGACAT	TGATGATTCC	TGGAAAAGTA	TAAAGAGTAT	CTTGGACTGG	ACATCTTTTA	780
ACCAGGAGAG	AATTGTTGAT	GTTGCTGGAC	CAGGGGGTTG	GAATGACCCA	GATATGTTAG	840
TGATTGGCAA	CTTTGGCCTC	AGCTGGAATC	AGCAAGTAAC	TCAGATGGCC	CTCTGGGCTA	900
TCATGGCTGC	TCCTTTATTC	ATGTCTAATG	ACCTCCGACA	CATCAGCCCT	CAAGCCAAAG	960
CTCTCATTCA	GGATAAGGAC	GTAATTGCCA	TCAATCAGGA	CCCCTTGGGC	AAGCAAGGGT	1020
ACCAGCTTAG	ACAGGGAGAC	AACTTTGAAG	TGTGGGAACG	ACCTCTCTCA	GGCTTAGCCT	1080
GGGCTGTAGC	TATGATAAAC	CGGCAGGAGA	TTGGTGGACC	TCGCTCTTAT	ACCATCGCAG	1140
TTGCTTCCCT	GGGTAAAGGA	GTGGCCTGTA	ATCCTGCCTG	CTTCATCACA	CAGCTCCTCC	1200
CTGTGAAAAG	GAAGCTAGGG	TTCTATGAAT	GGACTTCAAG	GTTAAGAAGT	CACATAAATC	1260
CCACAGGCAC	TGTTTTGCTT	CAGCTAGAAA	ATACAATGCA	GATGTCATTA	AAAGACTTAC	1320
TTTAAAAAAA	AAAAAACTC	GAG				1343

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 210 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTGGGCTGTA	GCTATGATAA	ACCGGCAGGA	GATTGGTGGA	CCTCGCTCTT	ATACCATCGC	60
AGTTGCTTCC	CTGGGTAAAG	GAGTGGCCTG	TAATCCTGCC	TGCTTCATCA	CACAGCTCCT	120
CCCTGTGAAA	AGGAAGCTAG	GGTCTATGA	ATGGACTTCA	AGGTTAAGAA	GTACATATAA	180
TCCCACAGGC	ACTGTTTTGC	TTCAGCTAGA				210

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 268 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

- 55 -

ATTGGTCCGC	CCCTGAGGTT	AATCTTAAAA	GCCCAGGTTA	CCCGCGGAAA	TTTATGCTGT	60
CCGGTCACCG	TGACAATGCA	GCTGAGGAAC	CCAGAACTAC	ATCTGGGCTG	CGCGCTTGCG	120
CTTCGCTTCC	TGGCCCTCGT	TTCCTGGGAC	ATCCCTGGGG	CTAGAGCACT	GGACAATGGA	180
TTGGCAAGGA	CGCCTACCAT	GGGCTGGCTG	CACTGGGAGC	GCTTCATGTG	CAACCTTGAC	240
TGCCAGGAAG	AGCCAGATTC	CTGCATCA				268

## (2) INFORMATION FOR SEQ ID NO:21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met	Ala	Thr	Gly	Ser	Arg	Thr	Ser	Leu	Leu	Leu	Ala	Phe	Gly	Leu	Leu
1				5				10					15		
Cys	Leu	Pro	Trp	Leu	Gln	Glu	Gly	Ser	Ala						
			20					25							

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGGCTACAG	GCTCCCGGAC	GTCCCTGCTC	CTGGCTTTTG	GCCTGCTCTG	CCGCCCCTGG	60
CTTCAAGAGG	GCAAGTGCC					78

## (2) INFORMATION FOR SEQ ID NO:23:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTTTGGATCC	CTCGAGGACA	TTGATTATTG	ACTAG	35
------------	------------	------------	-------	----

## (2) INFORMATION FOR SEQ ID NO:24:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TTTTGGATCC	CGTGTCAAGG	ACGGTGAC	28
------------	------------	----------	----

## (2) INFORMATION FOR SEQ ID NO:25:

## (i) SEQUENCE CHARACTERISTICS:

**SUBSTITUTE SHEET (RULE 26)**

- 56 -

(A) LENGTH: 1197 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ix) FEATURE:

(A) NAME/KEY: Coding Sequence  
 (B) LOCATION: 1...1194

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTG	GAC	AAT	GGA	TTG	GCA	AGG	ACG	CCT	ACC	ATG	GGC	TGG	CTG	CAC	TGG	48
Leu	Asp	Asn	Gly	Leu	Ala	Arg	Thr	Pro	Thr	Met	Gly	Trp	Leu	His	Trp	
1				5					10					15		
GAG	CGC	TTC	ATG	TGC	AAC	CTT	GAC	TGC	CAG	GAA	GAG	CCA	GAT	TCC	TGC	96
Glu	Arg	Phe	Met	Cys	Asn	Leu	Asp	Cys	Gln	Glu	Glu	Pro	Asp	Ser	Cys	
			20					25					30			
ATC	AGT	GAG	AAG	CTC	TTC	ATG	GAG	ATG	GCA	GAG	CTC	ATG	GTC	TCA	GAA	144
Ile	Ser	Glu	Lys	Leu	Phe	Met	Glu	Met	Ala	Glu	Leu	Met	Val	Ser	Glu	
		35					40					45				
GGC	TGG	AAG	GAT	GCA	GGT	TAT	GAG	TAC	CTC	TGC	ATT	GAT	GAC	TGT	TGG	192
Gly	Trp	Lys	Asp	Ala	Gly	Tyr	Glu	Tyr	Leu	Cys	Ile	Asp	Asp	Cys	Trp	
	50					55					60					
ATG	GCT	CCC	CAA	AGA	GAT	TCA	GAA	GGC	AGA	CTT	CAG	GCA	GAC	CCT	CAG	240
Met	Ala	Pro	Gln	Arg	Asp	Ser	Glu	Gly	Arg	Leu	Gln	Ala	Asp	Pro	Gln	
	65				70					75					80	
CGC	TTT	CCT	CAT	GGG	ATT	CGC	CAG	CTA	GCT	AAT	TAT	GTT	CAC	AGC	AAA	288
Arg	Phe	Pro	His	Gly	Ile	Arg	Gln	Leu	Ala	Asn	Tyr	Val	His	Ser	Lys	
				85				90						95		
GGA	CTG	AAG	CTA	GGG	ATT	TAT	GCA	GAT	GTT	GGA	AAT	AAA	ACC	TGC	GCA	336
Gly	Leu	Lys	Leu	Gly	Ile	Tyr	Ala	Asp	Val	Gly	Asn	Lys	Thr	Cys	Ala	
			100					105					110			
GGC	TTC	CCT	GGG	AGT	TTT	GGA	TAC	TAC	GAC	ATT	GAT	GCC	CAG	ACC	TTT	384
Gly	Phe	Pro	Gly	Ser	Phe	Gly	Tyr	Tyr	Asp	Ile	Asp	Ala	Gln	Thr	Phe	
		115					120					125				
GCT	GAC	TGG	GGA	GTA	GAT	CTG	CTA	AAA	TTT	GAT	GGT	TGT	TAC	TGT	GAC	432
Ala	Asp	Trp	Gly	Val	Asp	Leu	Leu	Lys	Phe	Asp	Gly	Cys	Tyr	Cys	Asp	
	130					135					140					
AGT	TTG	GAA	AAT	TTG	GCA	GAT	GGT	TAT	AAG	CAC	ATG	TCC	TTG	GCC	CTG	480
Ser	Leu	Glu	Asn	Leu	Ala	Asp	Gly	Tyr	Lys	His	Met	Ser	Leu	Ala	Leu	
	145				150					155					160	
AAT	AGG	ACT	GGC	AGA	AGC	ATT	GTG	TAC	TCC	TGT	GAG	TGG	CCT	CTT	TAT	528
Asn	Arg	Thr	Gly	Arg	Ser	Ile	Val	Tyr	Ser	Cys	Glu	Trp	Pro	Leu	Tyr	
				165					170					175		
ATG	TGG	CCC	TTT	CAA	AAG	CCC	AAT	TAT	ACA	GAA	ATC	CGA	CAG	TAC	TGC	576
Met	Trp	Pro	Phe	Gln	Lys	Pro	Asn	Tyr	Thr	Glu	Ile	Arg	Gln	Tyr	Cys	
			180					185					190			
AAT	CAC	TGG	CGA	AAT	TTT	GCT	GAC	ATT	GAT	GAT	TCC	TGG	AAA	AGT	ATA	624
Asn	His	Trp	Arg	Asn	Phe	Ala	Asp	Ile	Asp	Asp	Ser	Trp	Lys	Ser	Ile	
		195					200					205				
AAG	AGT	ATC	TTG	GAC	TGG	ACA	TCT	TTT	AAC	CAG	GAG	AGA	ATT	GTT	GAT	672
Lys	Ser	Ile	Leu	Asp	Trp	Thr	Ser	Phe	Asn	Gln	Glu	Arg	Ile	Val	Asp	
	210					215					220					

- 57 -

GTT GCT GGA CCA GGG GGT TGG AAT GAC CCA GAT ATG TTA GTG ATT GGC Val Ala Gly Pro Gly Gly Trp Asn Asp Pro Asp Met Leu Val Ile Gly 225 230 235 240	720
AAC TTT GGC CTC AGC TGG AAT CAG CAA GTA ACT CAG ATG GCC CTC TGG Asn Phe Gly Leu Ser Trp Asn Gln Gln Val Thr Gln Met Ala Leu Trp 245 250 255	768
GCT ATC ATG GCT GCT CCT TTA TTC ATG TCT AAT GAC CTC CGA CAC ATC Ala Ile Met Ala Ala Pro Leu Phe Met Ser Asn Asp Leu Arg His Ile 260 265 270	816
AGC CCT CAA GCC AAA GCT CTC CTT CAG GAT AAG GAC GTA ATT GCC ATC Ser Pro Gln Ala Lys Ala Leu Leu Gln Asp Lys Asp Val Ile Ala Ile 275 280 285	864
AAT CAG GAC CCC TTG GGC AAG CAA GGG TAC CAG CTT AGA CAG GGA GAC Asn Gln Asp Pro Leu Gly Lys Gln Gly Tyr Gln Leu Arg Gln Gly Asp 290 295 300	912
AAC TTT GAA GTG TGG GAA CGA CCT CTC TCA GGC TTA GCC TGG GCT GTA Asn Phe Glu Val Trp Glu Arg Pro Leu Ser Gly Leu Ala Trp Ala Val 305 310 315 320	960
GCT ATG ATA AAC CGG CAG GAG ATT GGT GGA CCT CGC TCT TAT ACC ATC Ala Met Ile Asn Arg Gln Glu Ile Gly Gly Pro Arg Ser Tyr Thr Ile 325 330 335	1008
GCA GTT GCT TCC CTG GGT AAA GGA GTG GCC TGT AAT CCT GCC TGC TTC Ala Val Ala Ser Leu Gly Lys Gly Val Ala Cys Asn Pro Ala Cys Phe 340 345 350	1056
ATC ACA CAG CTC CTC CCT GTG AAA AGG AAG CTA GGG TTC TAT GAA TGG Ile Thr Gln Leu Leu Pro Val Lys Arg Lys Leu Gly Phe Tyr Glu Trp 355 360 365	1104
ACT TCA AGG TTA AGA AGT CAC ATA AAT CCC ACA GGC ACT GTT TTG CTT Thr Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr Val Leu Leu 370 375 380	1152
CAG CTA GAA AAT ACA ATG CAG ATG TCA TTA AAA GAC TTA CTT TAA Gln Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp Leu Leu 385 390 395	1197

## (2) INFORMATION FOR SEQ ID NO:26:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 398 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Leu	Asp	Asn	Gly	Leu	Ala	Arg	Thr	Pro	Thr	Met	Gly	Trp	Leu	His	Trp
1				5					10					15	
Glu	Arg	Phe	Met	Cys	Asn	Leu	Asp	Cys	Gln	Glu	Glu	Pro	Asp	Ser	Cys
			20					25					30		
Ile	Ser	Glu	Lys	Leu	Phe	Met	Glu	Met	Ala	Glu	Leu	Met	Val	Ser	Glu
			35				40					45			
Gly	Trp	Lys	Asp	Ala	Gly	Tyr	Glu	Tyr	Leu	Cys	Ile	Asp	Asp	Cys	Trp
			50				55					60			

SUBSTITUTE SHEET (RULE 26)

- 58 -

```

Met Ala Pro Gln Arg Asp Ser Glu Gly Arg Leu Gln Ala Asp Pro Gln
65          70          75          80
Arg Phe Pro His Gly Ile Arg Gln Leu Ala Asn Tyr Val His Ser Lys
85          90          95
Gly Leu Lys Leu Gly Ile Tyr Ala Asp Val Gly Asn Lys Thr Cys Ala
100         105         110
Gly Phe Pro Gly Ser Phe Gly Tyr Tyr Asp Ile Asp Ala Gln Thr Phe
115         120         125
Ala Asp Trp Gly Val Asp Leu Lys Phe Asp Gly Cys Tyr Cys Asp
130         135         140
Ser Leu Glu Asn Leu Ala Asp Gly Tyr Lys His Met Ser Leu Ala Leu
145         150         155         160
Asn Arg Thr Gly Arg Ser Ile Val Tyr Ser Cys Glu Trp Pro Leu Tyr
165         170         175
Met Trp Pro Phe Gln Lys Pro Asn Tyr Thr Glu Ile Arg Gln Tyr Cys
180         185         190
Asn His Trp Arg Asn Phe Ala Asp Ile Asp Asp Ser Trp Lys Ser Ile
195         200         205
Lys Ser Ile Leu Asp Trp Thr Ser Phe Asn Gln Glu Arg Ile Val Asp
210         215         220
Val Ala Gly Pro Gly Gly Trp Asn Asp Pro Asp Met Leu Val Ile Gly
225         230         235         240
Asn Phe Gly Leu Ser Trp Asn Gln Gln Val Thr Gln Met Ala Leu Trp
245         250         255
Ala Ile Met Ala Ala Pro Leu Phe Met Ser Asn Asp Leu Arg His Ile
260         265         270
Ser Pro Gln Ala Lys Ala Leu Leu Gln Asp Lys Asp Val Ile Ala Ile
275         280         285
Asn Gln Asp Pro Leu Gly Lys Gln Gly Tyr Gln Leu Arg Gln Gly Asp
290         295         300
Asn Phe Glu Val Trp Glu Arg Pro Leu Ser Gly Leu Ala Trp Ala Val
305         310         315         320
Ala Met Ile Asn Arg Gln Glu Ile Gly Gly Pro Arg Ser Tyr Thr Ile
325         330         335
Ala Val Ala Ser Leu Gly Lys Gly Val Ala Cys Asn Pro Ala Cys Phe
340         345         350
Ile Thr Gln Leu Leu Pro Val Lys Arg Lys Leu Gly Phe Tyr Glu Trp
355         360         365
Thr Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr Val Leu Leu
370         375         380
Gln Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp Leu Leu
385         390         395

```

## (2) INFORMATION FOR SEQ ID NO:27:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 338 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```

ATGGCTACAG GTAAGCGCCC CTAAATCCC TTTGGGCACA ATGTGTCCTG AGGGGAGAGG      60
CAGCGACCTG TAGATGGGAC GGGGGCACTA ACCCTCAGGT TTGGGGCTTC TGAATGTGAG      120
TATCGCCATG TAAGCCCATG ATTTGGCCAA TCTCAGAAAG CTCCTGGTCC CTGGAGGGAT      180
GGAGAGAGAA AAACAAACAG CTCCTGGAGC AGGGAGAGTG CTGGCCTCTT GCTCTCCGGC      240
TCCCTCTGTT GCCCTCTGGT TTCTCCCCAG GCTCCCGGAC GTCCCTGCTC CTGGCTTTTG      300
GCCTGCTCTG CCTGCCCTGG CTTCAAGAGG GCAGTGCC      338

```

## (2) INFORMATION FOR SEQ ID NO:28:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid

- 59 -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Leu	Asp	Asn	Gly	Leu	Ala	Arg	Thr	Pro	Thr
1				5					10

- 60 -

What is claimed is:

1. A method of treatment comprising identifying a patient suspected of having an  $\alpha$ -galactosidase A deficiency, and introducing into the patient a human cell  
5 genetically modified to overexpress and secrete human  $\alpha$ -gal A.

2. The method of claim 1, wherein the cell has been transfected *in vitro* with a DNA molecule having a coding sequence encoding a polypeptide comprising human  
10  $\alpha$ -gal A (SEQ ID NO:26).

3. The method of claim 2, wherein the polypeptide further comprises an heterologous signal peptide.

4. The method of claim 3, wherein the heterologous signal peptide is the human growth hormone  
15 (hGH) signal peptide (SEQ ID NO:21).

5. The method of claim 3, wherein the DNA molecule comprises an intron within the sequence encoding the signal peptide.

6. The method of claim 3, wherein the DNA  
20 molecule comprises an untranslated sequence of at least 6 nucleotides lying immediately 3' to the termination codon of the coding sequence, said untranslated sequence comprising a polyadenylation site.

7. A DNA molecule comprising  
25 a first sequence encoding the hGH signal peptide (SEQ ID NO:21), said first sequence comprising an intron; and,

- 61 -

linked to the 3' end of the first sequence, a second sequence encoding a polypeptide comprising human  $\alpha$ -gal A.

8. The DNA molecule of claim 7, further  
5 comprising a 3' untranslated sequence comprising a polyadenylation site.

9. An expression construct comprising the DNA molecule of claim 7 and suitable for expression in a human cell.

10 10. A cultured human cell containing a DNA molecule that (a) encodes a polypeptide comprising human  $\alpha$ -gal A linked to an heterologous signal peptide, and (b) permits expression of the polypeptide in the cell.

11. The cell of claim 10, said cell being a  
15 fibroblast.

12. The cell of claim 10, wherein said cell is selected from the group consisting of an epithelial cell, an endothelial cell, a bone marrow cell, a glial cell, a hepatocyte, a keratinocyte, a myocyte, a neuron, or a  
20 precursor of these cell types.

13. The cell of claim 10, wherein said signal peptide is the hGH signal peptide (SEQ ID NO:21).

14. A clonal cell strain consisting essentially of a plurality of cultured human cells transfected with a  
25 DNA molecule that (a) encodes a polypeptide comprising human  $\alpha$ -gal A linked to an heterologous signal peptide, and (b) permits overexpression of the polypeptide in the cells.



- 62 -

15. The clonal cell strain of claim 14, said cells being fibroblasts.

16. A clonal cell line consisting essentially of a plurality of immortalized human cells transfected with  
5 a DNA molecule that (a) encodes a polypeptide comprising human  $\alpha$ -gal A linked to an heterologous signal peptide, and (b) permits overexpression of the polypeptide in the cells.

17. The clonal cell line of claim 16, wherein  
10 said cell is selected from the group consisting of a Bowes melanoma cell, a Daudi cell, a HeLa cell, an HL-60 cell, an HT1080 cell, a Jurkat cell, a KB carcinoma cell, a K-562 leukemia cell, an MCF-7 breast cancer cell, a MOLT-4 cell, a Namalwa cell, a Raji cell, an RPMI 8226  
15 cell, a U-937 cell, a WI-38VA13 (subline 2R4) cell, and a 2780AD ovarian carcinoma cell.

18. A protein comprising (a) the hGH signal peptide (SEQ ID NO:21) linked by a peptide bond to (b) human  $\alpha$ -gal A.

20 19. A process for making glycosylated human  $\alpha$ -gal A, comprising  
culturing the cell of claim 11 under conditions permitting expression of human  $\alpha$ -gal A from said DNA molecule and secretion of glycosylated human  $\alpha$ -gal A into  
25 the culture medium of the cell; and  
isolating glycosylated human  $\alpha$ -gal A from the culture medium.

20. Purified glycosylated human  $\alpha$ -gal A made by the process of claim 19.

- 63 -

21. A process for purifying human  $\alpha$ -gal A from a sample, comprising a first chromatography step wherein the sample is passed over a hydrophobic interaction resin.

5           22. The process of claim 21, wherein the functional moiety on the resin comprises a butyl group.

          23. A process for making glycosylated human  $\alpha$ -gal A, comprising  
          culturing the cell of claim 16 under conditions  
10   permitting expression of human  $\alpha$ -gal A from said DNA molecule and secretion of glycosylated human  $\alpha$ -gal A into the culture medium of the cell; and  
          isolating glycosylated human  $\alpha$ -gal A from the culture medium.

15           24. The process of claim 21, further comprising the step of passing the sample over a second resin selected from the group consisting of an immobilized heparin resin, hydroxyapatite, an anion exchange resin, and a size exclusion resin.

20           25. A method of treatment comprising identifying a patient suspected of having an  $\alpha$ -gal A deficiency, and  
          administering to the patient the purified glycosylated human  $\alpha$ -gal A of claim 20.

25           26. A therapeutic composition comprising pharmaceutically acceptable purified human  $\alpha$ -gal A having N-linked mannose-6-phosphate in a pharmaceutically acceptable carrier.

- 64 -

27. A therapeutic composition comprising the purified human  $\alpha$ -gal A of claim 20 and a pharmaceutically acceptable excipient.

28. The therapeutic composition of claim 27,  
5 formulated at pH 6.5 or lower.

29. The therapeutic composition of claim 28, wherein the pharmaceutically acceptable excipient is human serum albumin.

30. A DNA molecule encoding a protein comprising  
10 the signal peptide of hGH (SEQ ID NO:21) linked to a polypeptide other than hGH.

31. The DNA molecule of claim 30, wherein the DNA molecule comprises an intron within the sequence encoding the signal peptide.

15 32. A cultured human cell containing a DNA molecule that (a) encodes a polypeptide comprising human  $\alpha$ -gal A, and (b) permits overexpression of the polypeptide in the cell.

33. A process for making glycosylated human  
20  $\alpha$ -gal A, comprising culturing a human cell containing a DNA molecule that encodes a polypeptide comprising human  $\alpha$ -gal A under conditions permitting expression of human  $\alpha$ -gal A from said DNA molecule.

34. The therapeutic composition of claim 26  
25 further comprising a pharmaceutically acceptable excipient.

- 65 -

35. A process for purifying human  $\alpha$ -gal A from a sample comprising passing said sample over a hydrophobic interaction resin comprising a butyl group as a functional moiety.

1/11

CTGGGCTGTAGCTATGATAAACCGGCAGGA  
GATTGGTGGACCTCGCTCTTATACCATCGCA  
GTTGCTTCCCTGGGTAAAGGAGTGGCCTGTA  
ATCCTGCCTGCTTCATCACACAGCTCCTCCCT  
GTGAAAAGGAAGCTAGGGTTCTATGAATGGA  
CTTCAAGGTTAAGAAGTCACATAAATCCCAC  
AGGCACTGTTTTGCTTCAGCTAGA

**FIG. 1**

2/11

ATTGGTCCGCCCCTGAGGTTAATCTTAAAAG

SacII

CCCAGGTTACCCGCGGAAATTTATGCTGTC  
CGGTCACCGTGACAATGCAGCTGAGGAACC  
CAGAACTACATCTGGGCTGCGCGCTTGCGCT  
TCGCTTCCTGGCCCTCGTTTCCTGGGACATC  
CCTGGGGCTAGAGCACTGGACAATGGATTG

NcoI

GCAAGGACGCCTACCATGGGCTGGCTGCAC  
TGGGAGCGCTTCATGTGCAACCTTGACTGCC  
AGGAAGAGCCAGATTCCTGCATCA

**FIG. 2**

3/11

1 CCGCGGGAAA TTTATGCTGT CCGGTCACCG TGACAATGCA GCTGAGGAAC CCAGAACTAC  
61 ATCTGGGCTG CGCGCTTGCG CTTGCTTCC TGGCCCTCGT TTCCTGGGAC ATCCCTGGGG  
121 CTAGAGCACT GGACAATGGA TTGGCAAGGA CGCCTACCAT GGGCTGGCTG CACTGGGAGC  
181 GCTTCATGTG CAACCTTGAC TGCCAGGAAG AGCCAGATTC CTGCATCAGT GAGAAGCTCT  
241 TCATGGAGAT GGCAGAGCTC ATGGTCTCAG AAGGCTGGAA GGATGCAGGT TATGAGTACC  
301 TCTGCATTGA TGA CTGTTGG ATGGCTCCCC AAAGAGATTC AGAAGGCAGA CTTCAGGCAG  
361 ACCCTCAGCG CTTTCCTCAT GGGATTGCGC AGCTAGCTAA TTATGTTTAC AGCAAAGGAC  
421 TGAAGCTAGG GATTTATGCA GATGTTGGAA ATAAAACCTG CGCAGGCTTC CCTGGGAGTT  
481 TTGGATACTA CGACATTGAT GCCCAGACCT TTGCTGACTG GGGAGTAGAT CTGCTAAAT  
541 TTGATGGTTG TTA CTGTGAC AGTTTGGAAA ATTTGGCAGA TGGTTATAAG CACATGTCCT  
601 TGGCCCTGAA TAGGACTGGC AGAAGCATTG TGTACTCCTG TGAGTGGCCT CTTTATATGT  
661 GGCCCTTTCA AAAGCCCAAT TATACAGAAA TCCGACAGTA CTGCAATCAC TGGCGAAATT  
721 TTGCTGACAT TGATGATTCC TGGAAAAGTA TAAAGAGTAT CTTGGACTGG ACATCTTTTA  
781 ACCAGGAGAG AATTGTTGAT GTTGCTGGAC CAGGGGGTTG GAATGACCCA GATATGTTAG  
841 TGATTGGCAA CTTTGGCCTC AGCTGGAATC AGCAAGTAAC TCAGATGGCC CTCTGGGCTA  
901 TCATGGCTGC TCCTTTATTC ATGTCTAATG ACCTCCGACA CATCAGCCCT CAAGCCAAAG  
961 CTCTCCTTCA GGATAAGGAC GTAATTGCCA TCAATCAGGA CCCCTTGGGC AAGCAAGGGT  
1021 ACCAGCTTAG ACAGGGAGAC AACTTTGAAG TGTGGGAACG ACCTCTCTCA GGCTTAGCCT  
1081 GGGCTGTAGC TATGATAAAC CGGCAGGAGA TTGGTGGACC TCGCTCTTAT ACCATCGCAG  
1141 TTGCTTCCCT GGGTAAAGGA GTGGCCTGTA ATCCTGCCTG CTTTCATCACA CAGCTCCTCC  
1201 CTGTGAAAAG GAAGCTAGGG TTCTATGAAT GGA CTTC AAG GTTAAG AAGT CACATAAATC  
1261 CCACAGGCAC TGTTTTGCTT CAGCTAGAAA ATACAATGCA GATGTCATTA AAAGACTTAC  
1321 TTTAAAAAAA AAAAAA ACTC GAG

FIG. 3

4/11

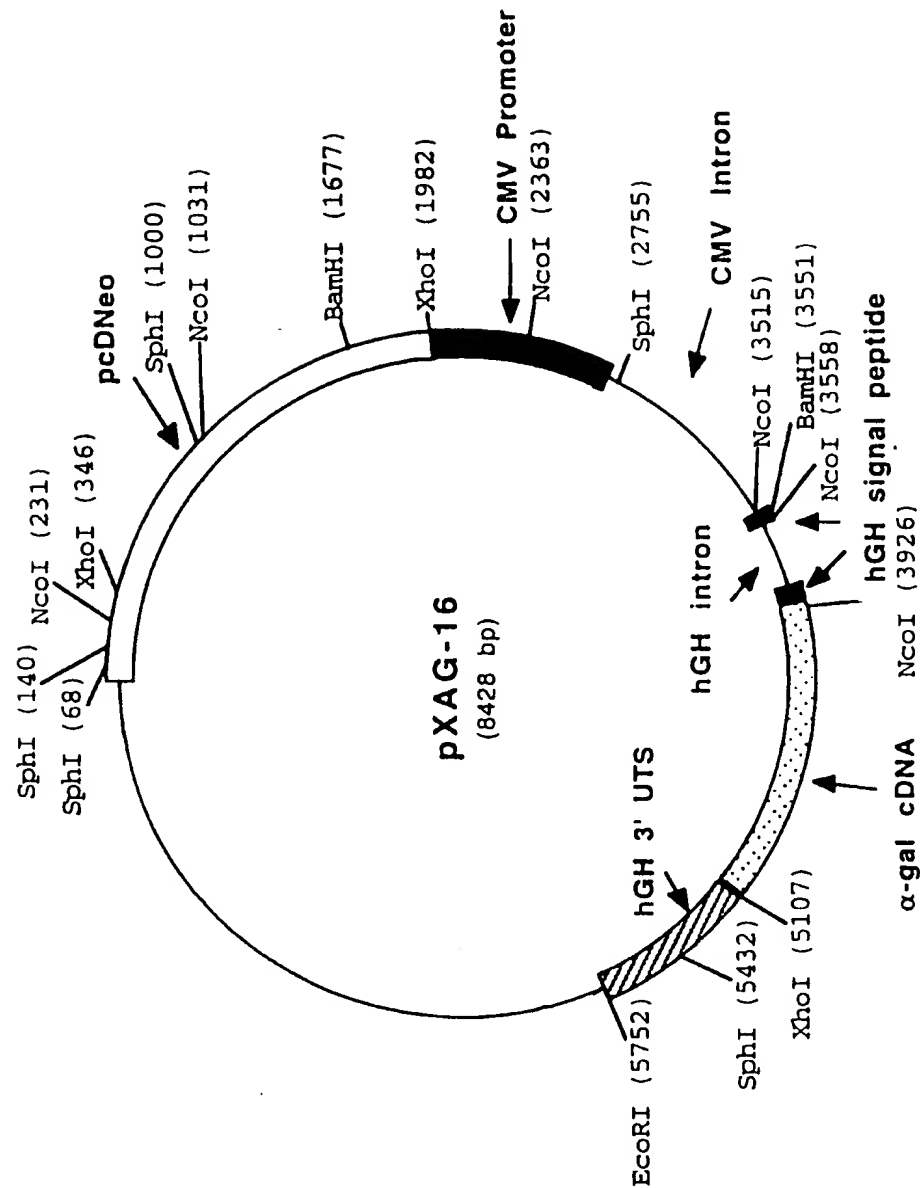


FIG. 4



5/11

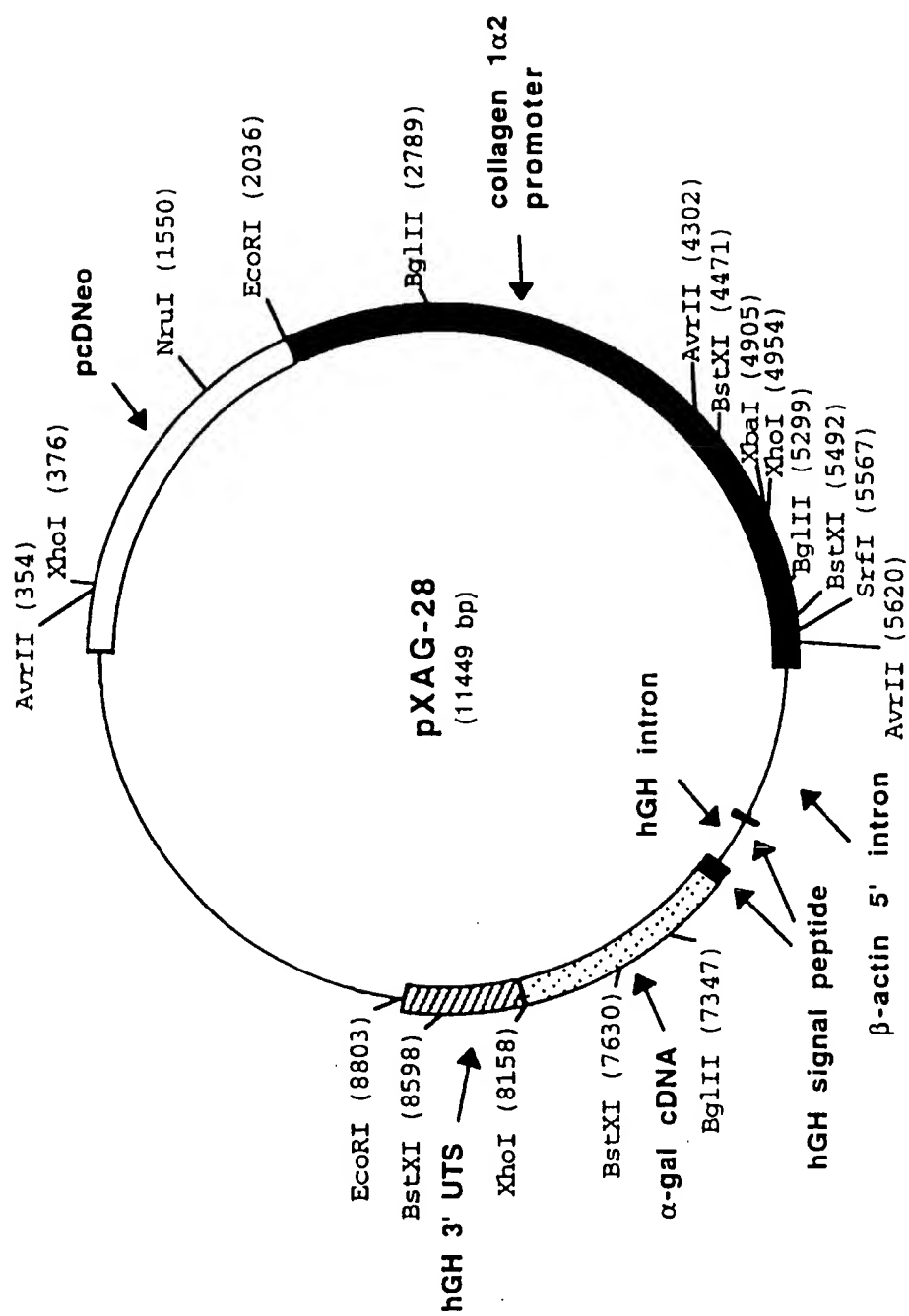


FIG. 5

6/11

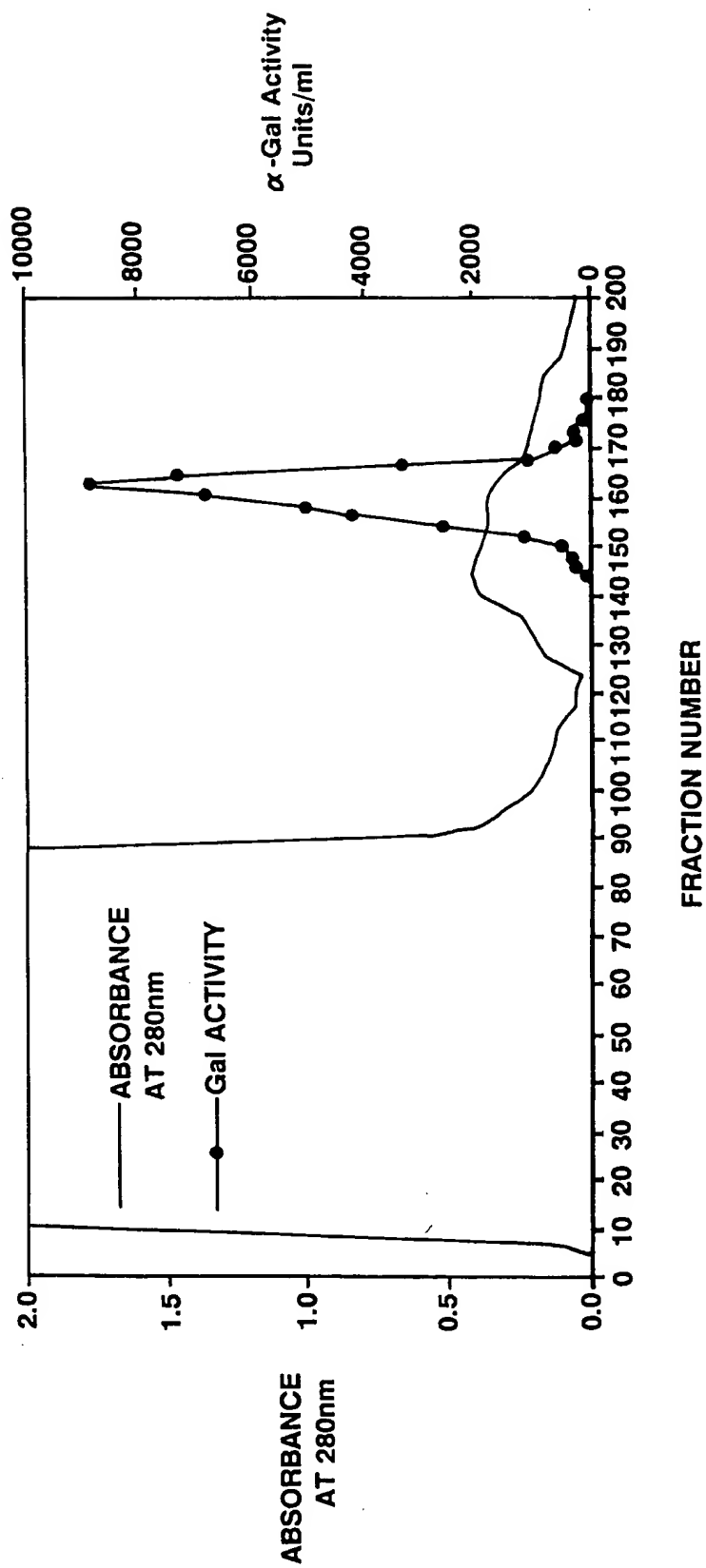


FIG. 6

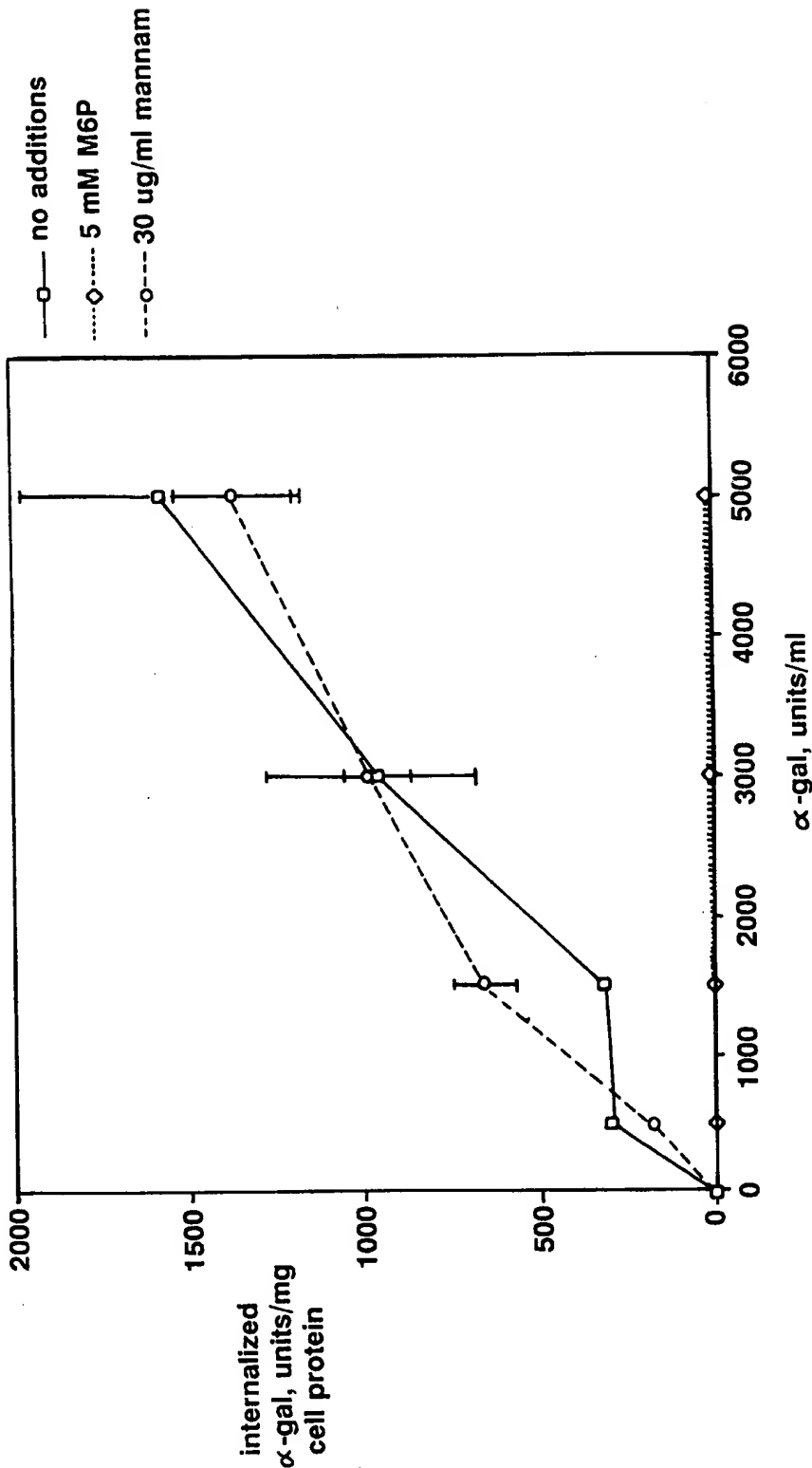


FIG. 7

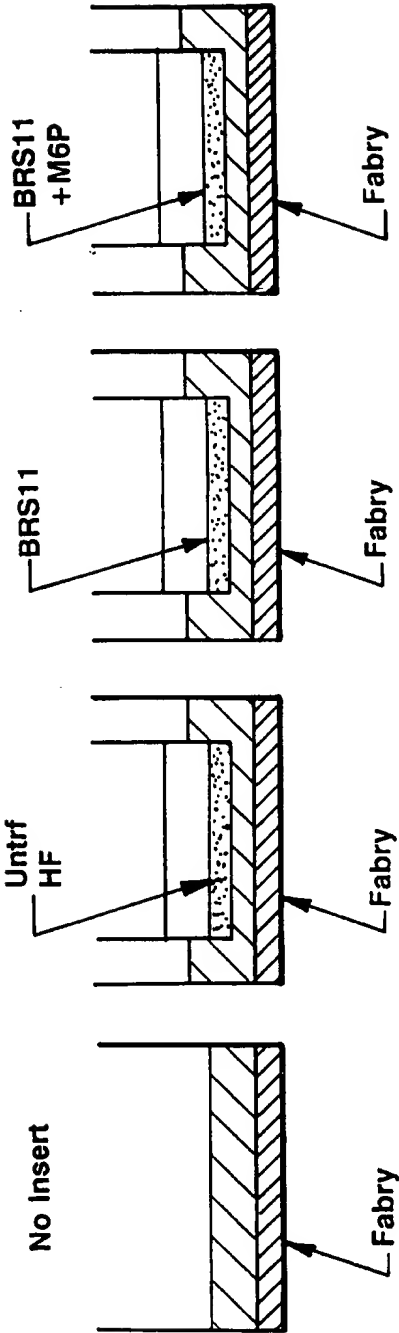


FIG. 8

9/11

Leu	Asp	Asn	Gly	Leu	Ala	Arg	Thr	Pro	Thr	Met	Gly
Trp	Leu	His	Trp	Glu	Arg	Phe	Met	Cys	Asn	Leu	Asp
Cys	Gln	Glu	Glu	Pro	Asp	Ser	Cys	Ile	Ser	Glu	Lys
Leu	Phe	Met	Glu	Met	Ala	Glu	Leu	Met	Val	Ser	Glu
Gly	Trp	Lys	Asp	Ala	Gly	Tyr	Glu	Tyr	Leu	Cys	Ile
Asp	Asp	Cys	Trp	Met	Ala	Pro	Gln	Arg	Asp	Ser	Glu
Gly	Arg	Leu	Gln	Ala	Asp	Pro	Gln	Arg	Phe	Pro	His
Gly	Ile	Arg	Gln	Leu	Ala	Asn	Tyr	Val	His	Ser	Lys
Gly	Leu	Lys	Leu	Gly	Ile	Tyr	Ala	Asp	Val	Gly	Asn
Lys	Thr	Cys	Ala	Gly	Phe	Pro	Gly	Ser	Phe	Gly	Tyr
Tyr	Asp	Ile	Asp	Ala	Gln	Thr	Phe	Ala	Asp	Trp	Gly
Val	Asp	Leu	Leu	Lys	Phe	Asp	Gly	Cys	Tyr	Cys	Asp
Ser	Leu	Glu	Asn	Leu	Ala	Asp	Gly	Tyr	Lys	His	Met
Ser	Leu	Ala	Leu	Asn	Arg	Thr	Gly	Arg	Ser	Ile	Val
Tyr	Ser	Cys	Glu	Trp	Pro	Leu	Tyr	Met	Trp	Pro	Phe
Gln	Lys	Pro	Asn	Tyr	Thr	Glu	Ile	Arg	Gln	Tyr	Cys
Asn	His	Trp	Arg	Asn	Phe	Ala	Asp	Ile	Asp	Asp	Ser
Trp	Lys	Ser	Ile	Lys	Ser	Ile	Leu	Asp	Trp	Thr	Ser
Phe	Asn	Gln	Glu	Arg	Ile	Val	Asp	Val	Ala	Gly	Pro
Gly	Gly	Trp	Asn	Asp	Pro	Asp	Met	Leu	Val	Ile	Gly
Asn	Phe	Gly	Leu	Ser	Trp	Asn	Gln	Gln	Val	Thr	Gln
Met	Ala	Leu	Trp	Ala	Ile	Met	Ala	Ala	Pro	Leu	Phe
Met	Ser	Asn	Asp	Leu	Arg	His	Ile	Ser	Pro	Gln	Ala
Lys	Ala	Leu	Leu	Gln	Asp	Lys	Asp	Val	Ile	Ala	Ile
Asn	Gln	Asp	Pro	Leu	Gly	Lys	Gln	Gly	Tyr	Gln	Leu
Arg	Gln	Gly	Asp	Asn	Phe	Glu	Val	Trp	Glu	Arg	Pro
Leu	Ser	Gly	Leu	Ala	Trp	Ala	Val	Ala	Met	Ile	Asn
Arg	Gln	Glu	Ile	Gly	Gly	Pro	Arg	Ser	Tyr	Thr	Ile
Ala	Val	Ala	Ser	Leu	Gly	Lys	Gly	Val	Ala	Cys	Asn
Pro	Ala	Cys	Phe	Ile	Thr	Gln	Leu	Leu	Pro	Val	Lys
Arg	Lys	Leu	Gly	Phe	Tyr	Glu	Trp	Thr	Ser	Arg	Leu
Arg	Ser	His	Ile	Asn	Pro	Thr	Gly	Thr	Val	Leu	Leu
Gln	Leu	Glu	Asn	Thr	Met	Gln	Met	Ser	Leu	Lys	Asp
Leu	Leu										

FIG. 9

10/11

ATGGCTACAG GTAAGCGCCC CTAAAATCCC TTGGGCACA  
ATGTGTCCTG AGGGGAGAGG CAGCGACCTG TAGATGGGAC  
GGGGGCACTA ACCCTCAGGT TTGGGGCTTC TGAATGTGAG  
TATCGCCATG TAAGCCCAGT ATTTGGCCAA TCTCAGAAAG  
CTCCTGGTCC CTGGAGGGAT GGAGAGAGAA AAACAAACAG  
CTCCTGGAGC AGGGAGAGTG CTGGCCTCTT GCTCTCCGGC  
TCCCTCTGTT GCCCTCTGGT TTCTCCCCAG GCTCCCGGAC  
 GTCCCTGCTC CTGGCTTTTG GCCTGCTCTG CCTGCCCTGG  
 CTTCAAGAGG GCAGTGCC

**FIG. 10**

ATGGCTACAG GCTCCCGGAC GTCCCTGCTC CTGGCTTTTG  
 GCCTGCTCTG CCTGCCCTGG CTTCAAGAGG GCAGTGCC

**FIG. 11**

Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala  
 Phe Gly Leu Leu Cys Leu Pro Trp Leu Gln Glu Gly  
 Ser Ala

**FIG. 12**

11/11

```

1  CTGGACAATG GATTGGCAAG GAGCCCTACC ATGGGCTGGC TGCACTGGGA GCGCTTCATG
61  TGCAACCTTG ACTGCCAGGA AGAGCCAGAT TCCTGCATCA GTGAGAAAGCT CTTTCATGGAG
121  ATGGCAGAGC TCATGGTCTC AGAAGGCTGG AAGGATGCAG GTTATGAGTA CCTCTGCATT
181  GATGACTGTT GGATGGCTCC CCAAAGAGAT TCAGAAAGGCA GACTTCAGGC AGACCCTCAG
241  CGCTTTCCTC ATGGGATTGG CCAGCTAGCT AATTATGTTT ACAGCAAAGG ACTGAAGCTA
301  GGGATTATG CAGATGTTGG AAATAAAACC TGCCAGGCT TCCCTGGGAG TTTTGGATAC
361  TACGACATTG ATGCCCAGAC CTTTGCTGAC TGGGAGTAG ATCTGCTAAA ATTGATGGT
421  TGTTACTGTG ACAGTTTGGG AAATTTGGCA GATGTTATA AGCACATGTC CTTGGCCCTG
481  AATAGGACTG GCAGAAAGCAT TGTGACTCC TGTGAGTGGC CTCCTTATAT GTGGCCCTTT
541  CAAAAGCCCA ATTATACAGA AATCCGACAG TACTGCAATC ACTGGCGAAA TTTTGCTGAC
601  ATTGATGATT CCTGGAAAAG TATAAGAGT ATCTTGGACT GGACATCTTT TAACCAGGAG
661  AGAATTGTTG ATGTTGCTGG ACCAGGGGGT TGGAAATGACC CAGATATGTT AGTGATTGGC
721  AACTTTGGCC TCAGCTGGAA TCAGCAAGTA ACTCAGATGG CCTCTGGGC TATCATGGCT
781  GCTCCTTTAT TCATGTCTAA TGACCTCCGA CACATCAGCC CTCAAAGCCAA AGCTCTCCTT
841  CAGGATAAGG ACGTAATTGC CATCAATCAG GACCCCTTGG GCAAGCAAAGG GTACCAGCTT
901  AGACAGGGAG ACAACTTTGA AGTGTGGGAA CGACCTCTCT CAGGCTTAGC CTGGGCTGTA
961  GCTATGATAA ACCGGCAGGA GATTGGTGGG CTTGCTCTT ATACCATCGC AGTTGCTTCC
1021  CTGGGTAAAG GAGTGGCCTG TAATCCTGCC TGCTTCATCA CACAGCTCCT CCTGTGAAA
1081  AGGAAGCTAG GGTTCCTATG ATGGACTTCA AGGTTAAGAA GTCACATAAA TCCACACAGG
1141  ACTGTTTTGC TTCAGCTAGA AAATACAATG CAGATGTCAT TAAAAGACTT ACTTTAA

```

FIG. 13